Calcium Extrusion Mechanisms and Dendritic Development of Cerebellar Purkinje Cells

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Dekan
Prof. Martin Spiess

..... dedicated to my late father Dagadu Ranu Sherkhane (1919-2007) and brother Milind Dagadu Sherkhane (1964-2004).

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LIST OF ABBREVIATIONS

Ca^{2+}	calcium
PMCA2	Plasma membrane Ca^{2+} ATPase, Isoform 2
Na^{+}	Sodium
NCX	Na^{+}/Ca^{2+}exchanger
CB6	B6CF1
Carboxyeosin	CEDA-SE (5-(and-6)-Carboxyeosin diacetate succinimidyl Ester
DHPG	(*S*)-3,5-Dihydroxyphenylglycine
mGluR1	metabotropic glutamate receptor, type 1
PMA	phorbol-12-myristate-13-acetate
IR	Immunoreactivity
Bepridil	*N*-Benzyl-*N*-(3-isobutoxy-2-pyrrolidin-1-yl-propyl) aniline hydrochloride
KB-R7943	2-[4-[(4-nitrophenyl)methoxy]phenyl]ethyl ester carbamimidothioic acid methanesulfonate
CB-DMB	3-amino-6-chloro-5-[(4-chloro-benzyl) amino]-N [[(2,4dimethylbenzyl) amino]iminomethyl]-pyrazinecarboxamide
ORM-10103	2-[(3, 4-Dihydro-2-phenyl-2H-1-benzopyran-6-yl) oxy]-5-nitro-pyridine
SEA0400	2-[4-[(2, 5-difluorophenyl)methoxy] phenoxy]-5-ethoxyaniline
YM-244769	*N*-[(3-Aminophenyl) methyl]-6-[4-[(3-fluorophenyl)methoxy]phenoxy]-3 pyridinecarboxamide dihydrochloride
SN-6	2-[[4-[(4-Nitrophenyl) methoxy] phenyl]methyl]-4-thiazolidinecarboxylic acid ethyl ester
CNQX	6-Cyano-7-nitroquinoxaline-2, 3- dione
DL-AP5	DL-2-Amino-5-phosphonopentanoic acid
Gabazine	2-(3-Carboxypropyl)-3-amino-6-(4 methoxyphenyl) pyridazinium bromide
DIV	Days in vitro
BMP-7	bone morphogenetic protein-7
OP-1	osteogenic protein-1
Cpg15	candidate plasticity gene 15
Eph receptors	erythropoietin-producing human hepatocellular receptors
NMDA	N-methyl-D-aspartate receptor
CRF	corticotropin-releasing factor
$GABA_A$	gamma-Aminobutyric acid $receptor_A$
Sema 3A	semaphorin 3A
Op18	oncoprotein18
AMPA	(RS)-a-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)
BDNF	brain-derived neurotrophic factor

CF	climbing fiber
CGN	cerebellar granule cell
CNS	central nervous system
DCN	deep cerebellar neuron
EtD	a 1:1 mixture of ethanol and dimethyl sulfoxide
GluRδ2	glutamate receptor δ2
IGL	internal granule layer
LTD	long term depression
ML	molecular layer
MW	molecular weight
mwk	moonwalker
NGF	nerve growth factor
NT	neurotrophin
P	postnatal day
P/Q-block	combination of 100 nM ω-agatoxin IVA and 1 µM ω-conotoxin MVIIC
PTN	pleiotrophin
PTPζ	protein tyrosine phosphatase zeta
PB	phosphate buffer
PC	Purkinje cell
PCL	Purkinje cell layer
PF	parallel fiber
PKC	protein kinase C
PLC	phospholipase C
PM	preparation medium
RL	rhombic lip
ROR α	retinoid-related orphan receptor α
SCA	spinocerebellar ataxia
SS	stock solution
TF	transcription factor
$TRPC_3$	transient receptor potential cation channel, type 3
VGCC	voltage gated calcium channel

Summary

The cerebellum plays a vital role in motor learning and refining the motor activity. Many cell components in the cerebellum synchronize and process motor function, with Purkinje cells and granule cells being the key players in cerebellar circuitry. In particular, Purkinje cells are the principal neurons of the cerebellar cortex and are well known for their profuse and elaborate dendritic arbour. Previously, our lab has shown that the activity of Protein kinase C and the metabotropic glutamate receptor mGluR1 severely inhibits growth and development of Purkinje cell dendritic arbour (Metzger and Kapfhammer, 2000; Schrenk et al., 2002; Sirzen-Zelenskaya et al., 2006). The dendritic reduction caused by the activation of mGluR1 and PKC is partially rescued by the blockade of P/Q and T-type of Ca^{2+} channels (Gugger et al., 2012). Besides the Ca^{2+} influx, Ca^{2+} extrusion mechanisms also play an important role in Purkinje cell dendritic development (Huang et. al., 2010; Kim et al., 2007; Chris et al., 2013).

In my thesis, I have studied two plasma membrane antiporters that are involved in modulating calcium equilibrium in Purkinje cell dendritic development. The plasma membrane Ca^{2+}-ATPase2 (PMCA2) is involved in the extrusion of calcium and cerebellar synapse function. Of the 4 known PMCA variants, PMCA1 and PMCA4 are expressed ubiquitously whereas PMCA2 and PMCA3 are expressed prevalently in the central nervous systems. The PMCA2 isoform is highly expressed in the cerebellum, particularly in Purkinje cell dendrites and dendritic spines. By immunohistochemistry, we confirmed that PMCA2 immunoreactivity (IR) was strongly expressed at the dendritic plasma membrane and in dendritic spines of Purkinje cells. The chronic functional inhibition of PMCA2 by carboxyeosin in cerebellar slice cultures resulted in a slight reduction of the Purkinje cell dendritic arbor. On the other hand, chronic activation of mGluR1 by DHPG induced a strong reduction of the Purkinje cell dendritic tree. With co-treatment of an mGluR1 agonist and PMCA2 antagonist, PMCA2 functional inhibition surprisingly had a partial rescuing effect for the DHPG induced reduction of Purkinje cell dendritic development, indicating that PMCA2 plays an important role in calcium homeostasis controlling Purkinje cell dendritic growth and development. These finding suggest that PMCA2 is important for the maintenance and control of the calcium equilibrium in developing Purkinje cell dendrites and that this equilibrium is critical for the control of the dendritic growth and expansion.

The Na^{+}/Ca^{2+} exchanger (NCX) is another calcium extrusion mechanism in Purkinje cells that mediates Ca^{2+} and Na^{+} fluxes across the plasma membrane in a so-called bi-directional mode; the forward mode and the reverse mode. NCX has three isoforms, NCX1–3, and all of them are expressed

in Purkinje cells. NCX2 and NCX3 are predominantly expressed in the Purkinje cell soma, but NCX1 being the most abundant was expressed in the cell soma, stem dendrite and distal dendrites. The expression of NCX was not only restricted to Purkinje cells, but ubiquitous in the cerebellum particularly in the cytoplasm of cerebellar granule cells and molecular interneurons like basket cells and stellate cells.

The pharmacological blockade of the forward mode of NCX (Ca^{2+} efflux mode) by bepridil moderately inhibited growth and development of the Purkinje cell dendritic arbor in cerebellar slice cultures. However, the blockade of the reverse mode (Ca^{2+} influx mode) by KB-R7943 severely reduced the dendritic arbour and induced a morphological change with thickened distal dendrites. We used a number of additional NCX inhibitors like CB-DMB, ORM-10103, SEA0400, YM-244769 and SN-6 which have higher specificity for NCX isoforms and target either the forward, reverse or both modes. All these inhibitors produced a strong dendritic reduction similar to that seen with KB-R7943 without producing thickened distal dendrites. This indicates that effect of KB-R7943 on the thickened distal dendritic morphology was fairly unspecific and unrelated to the function of NCX. When KB-R7943 was combined with the antagonists of voltage gated calcium channels, the dendritic reduction was consistent and also apparent in the absence of bioelectrical activity indicating that it was mediated by NCX expressed in Purkinje cells. The pharmacological treatments also seem to have affected the morphology and number of dendritic spines on the Purkinje cell dendritic arbor.

Our findings indicate that the disturbance of the NCX-dependent calcium transport in Purkinje cells induces changes in the calcium handling of Purkinje cells causing dendritic reduction. Further, they underline the importance of the calcium equilibrium for the development and growth of the dendritic arbour in cerebellar Purkinje cells.

1. INTRODUCTION

1.1. Background

The human brain is probably the most complex living structure, with an estimated 100 billion neurons communicating with each other by as many as 1,000 trillion synaptic connections. Humans are known for sporting a bigger brain compared to other primates. The complexity of human brain has developed and evolved over a period of 6 million years and its size has tripled in past 2 million years. The brain is the principal structure of the central nervous system.

The nervous system is composed of vast number of neurons with characteristic afferent and efferent projections and dendritic morphologies and molecular identities. The development of the nervous system proceeds in several stages. Neurons are born, they extend and migrate to their final destinations in the nervous system. Further, they elaborate axons and dendrites in a characteristic pattern defining specific cell types. Finally, highly specific synaptic connections between neurons are made (AK McAllister, 2000).

Synapse formation involves two partners, axons and dendrites. The axon of a presynaptic neuron needs to be properly guided to the correct targets, which are usually the dendrites of the post-synaptic neurons. Most likely, synapse formation involves a two-way communication between the pre-synaptic cell and the post-synaptic cell (Jan and Jan, 2001).

The dendritic branching pattern varies to a great extent with the neuronal type, and is an important determinant of the synaptic input received by a neuron (Stuart et al., 2000). Dendrites are the sites for most of the synaptic connections and dendritic development determines the number and pattern of synapses received by each neuron (Hume ad Purves, 1981; Purves and Hume, 1981; Purves et al., 1986). Abnormalities in dendritic growth can have a profound impact and result in neurodevelopmental disorders such as mental retardation (Purpura, 1975). Genetic disorders with well-defined dendritic anomalies involving dendritic branching and/or spine formation include Down, Rett and Fragile-X syndromes (Kaufmann and Moser, 2000). Therefore, the proper dendritic growth, branching, arborisation and dendritic spine development are crucial for the functioning of the nervous system.

1.1.1. General dendritic development

Dendrites are the primary site for synapse formation in the vertebrate nervous system, and neurons that are deficient of dendrites receive less synaptic inputs than cells with complex dendritic arbours (Purves, 1988). Therefore, it's important to understand how the number of afferent synapses is

determined and it's necessary to identify the molecules that regulate the dendritic growth (Lein et al., 1995). Dendritic growth is considered to occur in two phases: initial extension followed by elongation and ramification. Many molecules, including neurotransmitters, hormones and neurotrophic factors have been shown to modulate the expansion of the dendritic arbour (Kelly, 1988; Mattson, 1988; Snider, 1988). Molecules that influence and affect dendritic expansion, growth and development will be discussed in the following section.

1.1.2. Molecules implicated in regulation of dendritic outgrowth and orientation

i. Bone morphogenetic protein-7

Osteogenic protein-1 (OP-1), also known as bone morphogenetic protein-7 (BMP-7), is a member of the transforming growth factor β (TGFβ) superfamily (Sampath et al., 1992; Sampath and Rueger, 1994). OP-1 is expressed in the developing nervous system and it has been found to induce dendritic growth in sympathetic neurons (Guo et al., 1998). OP-1, BMP-2, BMP-6 and *Drosophila* 60A induce dendritic growth in rat sympathetic ganglion neurons in a concentration-dependent manner with upregulation of the microtubule associated protein, MAP2 (Guo et al., 1998). Sympathetic neurons in rats extend an axon but no dendrites when maintained in culture in the absence of glia and serum. Exposure to OP-1 induces the formation of dendrites in these cultured neurons. OP-1 requires nerve growth factor (NGF) as a cofactor and, in the presence of optimal concentrations of NGF, OP- 1-induced dendritic growth from cultured perinatal neurons is comparable to that observed in situ. However, OP-1 can also induce dendrite growth from naïve neurons derived from 14.5-day embryos, suggesting that OP-1 is capable of promoting de novo formation of dendrites as opposed to merely promoting dendritic regeneration. The action of OP-1 is likely to be specific for dendrites as it has no obvious effect on axon numbers (Lein et al., 1995). OP-1 has also proven to stimulate dendritic growth in cultured cortical neurons (Le Roux et al., 1999).

ii. Semaphorins

Semaphorins are a family of cell-surface and soluble proteins that are able to regulate cell–cell interactions as well as cell differentiation, morphology and function. Semaphorins (also known as collapsins) were originally identified by their ability to collapse or repel axon growth cones (Luo et al., 1993; Messersmith et al., 1995). In mammals, 20 semaphorins have been identified and divided into five classes (semaphorins 3–7) that are characterized by particular structural properties (SNC, 1999).

Most of the effects of semaphorins are mediated by plexins and both are widely expressed in the mammalian CNS. Class A plexins are directly activated by several membrane-bound class 5 and class 6 semaphorins, whereas their activation by secreted class 3 semaphorins requires neuropilins as co-receptors to stabilize the semaphorin–plexin interaction. Class B plexins are activated by class 4 and class 5 semaphorins, and plexin C1 is the receptor for SEMA7A. Plexin D1 binds several class 3 semaphorins in a neuropilin dependent manner and can bind SEMA3E and SEMA4A independently of neuropilins. The semaphorin–plexin complex appears to be involved in multiple functions during development, for example in the nervous system, the immune system and during angiogenesis (Pasterkamp RJ, 2012; Gu & Giraudo, 2013; Neufeld et al., 2012; Kumanogoh and Kikutani, 2013).

Semaphorin 3A (Sema 3A) evidently is the best studied example of an axon guidance molecule that influences dendritic development. Sema 3A functions as a chemo-attractant for cortical apical dendrites of pyramidal neurons, in contrast it has a chemo-repellent action on cortical axons (Polleux et al., 2000).

Pyramidal neurons in the cerebral cortex normally have their apical dendrites extending toward the pial surface influenced by a diffusible factor, probably Sema 3A (Polleux et al., 2000). The difference in the effects of Sema 3A on axons and dendrites is due to the asymmetric localization of soluble guanylate cyclase (SGC) in axons and dendrites. Interestingly, apical dendrites express high levels of SGC and cGMP signalling appears to be necessary for the pial-directed orientation of dendritic growth. Thus, the differential effect of Sema 3A on axons and dendrites is most likely mediated by the asymmetric localization of intracellular signal molecules such as SGC (Polleux et al., 2000).

iii. Rho related GTPases

The small GTPases of the Rho subfamily are critical regulators of the actin cytoskeleton in eukaryotic cells from yeast to humans. Many studies suggest that Rho GTPases are involved in the regulation of neuronal morphogenesis, including migration, polarity, axon growth and guidance, dendrite elaboration and plasticity, and synapse formation (Luo, 2000). Investigations in intracellular effectors of dendritic development have revealed that dendritic growth and branching are differentially affected by activation of the Rho-family GTPases, RhoA, Rac1, and Cdc42 (Redmond and Ghosh 2001).

In rat hippocampal slice cultures, Rac and Rho play distinct functions in regulating dendritic spines and branches and are vital for the maintenance and reorganization of dendritic structures in maturing pyramidal neurons (Nakayama et al., 2000). Dominant-negative mutations of Rac1 lead to a marked

decrease in the number of primary dendrites, suggesting that endogenous Rac1 is an important effector of dendrite initiation. Inhibition of Cdc42 also leads to a reduction in the number of primary dendrites, suggesting that dendrite initiation may be mediated by a common effector of Rac1 and Cdc42 (Redmond and Ghosh 2001).

In cortical neurons, expression of dominant negative mutants of Rac or Cdc42, the Rho-inhibitory molecule C3 transferase or the GTPase-activating protein RhoGAP p190 causes a marked reduction in the number of primary dendrites in multipolar neurons and in the number of basal dendrites in neurons with pyramidal morphologies (Threadgill et al., 1997).

Together with the Rho family of small GTPases, other regulators of the cytoskeleton are implicated in the control of dendritic branching, including the Drosophila gene *kakapo*. The *kakapo* or *short stop* mutants are defective in dendritic branching of both sensory neurons (Gao et al. 1999) and motor neurons (Prokop et al. 1998).

iv. Ephrins

Ephrins are a family of proteins that function as the ligands for the Eph receptors which are the largest known subfamily of receptor protein-tyrosine kinases (RTKs). The ephrins and Eph receptors are implicated as positional labels that may guide the development of neural topographic maps, in pathway selection by axons, the guidance of cell migration and the establishment of regional patterns in the nervous system (Flanagan and Vanderhaeghen, 1998). Ephrins appear to serve typically, as repellents throughout development to influence axon pathfinding and topographic mapping, as well as restricting cell migration and intermingling (O'Leary and Wilkinson, 1999). In addition to their role in axon guidance, ephrins might also influence the dendritic development of pyramidal neurons in visual cortex. Transfection of cultured pyramidal neurons from ferret visual cortical slices with EphA3 receptor decreases branching of both apical and basal dendrites in transfected neurons (Butler et al., 1999).

v. Cpg15

Candidate plasticity gene 15 (cpg15) was identified in a forward genetic approach designed to isolate activity regulated genes that mediate synaptic plasticity (Nedivi et al., 1993). Cpg15 is predicted by the sequence analysis to be a membrane bound protein that has glycosyl-phosphatidyl-inositol linkage (Naeve et al., 1997). Interestingly, Cpg15 encodes an activity-regulated molecule that can promote dendritic growth. Cpg15 expressed in areas undergoing afferent innervation, dendritic elaboration

and synapse formation during development (Corriveau et al., 1999). In vivo transfection of Cpg15 in Xenopus tectum enhanced dendritic arbour growth exclusively in projection neurons, without affecting interneurons (Nedivi et al., 1998). Therefore, Nedivi and colleagues underlined that Cpg15 may represent a new class of activity-regulated, membrane-bound, growth promoting proteins that permit exquisite spatial and temporal control of neuronal plasticity.

1.2. Cerebellum

The cerebellum (meaning "little brain") is located at the dorsal side of the brain overlying the dorsal aspect of the rhombencephalon. The cerebellum accounts for approximately 10% of total brain mass and contains up to 50% of the total neurons in the brain. It has also been termed as a "neuronal machine" because of its precise architecture and machine-like neuronal circuitry (Eccles, 1967, Ito, 2006).

The cerebellum has two hemispheres which are separated by a midline part called vermis. Quite similar to the cerebral cortex the cerebellum consists of grey matter and white matter:

- The Grey matter is located on the surface of the cerebellum, forming the cerebellar cortex which is made of tightly folded layers. Each ridge of the layer is called a follium.
- On the other hand, the white matter is located underneath the cerebellar cortex, made up of mostly myelinated nerve fibres, and bearing four deep cerebellar nuclei embedded in it.

The cerebellum can be subdivided in to three ways – anatomical lobes, zones, and functional divisions (shown in Fig. 1). **The anatomical lobes** can be distinguished as the anterior lobe, the posterior lobe and the flocculonodular lobe. These lobes are divided by two fissures – the **primary fissure** and **posterolateral fissure**. There are three cerebellar zones, the midline zone is the **vermis**. On either side of the vermis is the **intermediate zone**. Lateral to the intermediate zone are the **lateral hemispheres**.

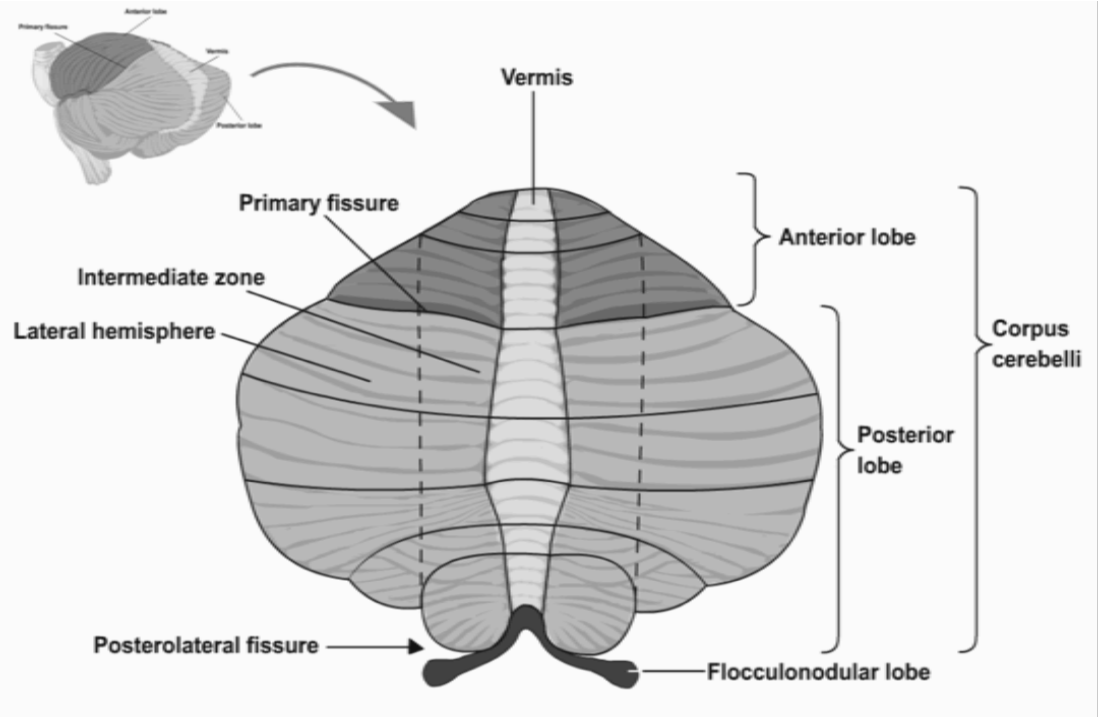
1.2.1 Functional subdivisions of the cerebellum: The cerebellum is divided into three functional areas – the cerebrocerebellum, the spinocerebellum and the vestibulocerebellum.

i. The Cerebrocerebellum is the largest division, formed by the two lateral regions of cerebellar hemispheres. The cerebrocerebellum communicates with the cerebral cortex via pontine nuclei and thalamus. It is thought to be involved in cognitive function, planning movements and motor learning. This area also controls co-ordination of muscle activation and is important in visually guided movements.

ii. The Spinocerebellum is located in the medial region of the cerebellum, comprised of the vermis and intermediate zone of the cerebellar cortex. The spinocerebellum receives somatosensory inputs from the spinal cord. The spinocerebellum is important in regulating the muscle tone, maintain balance and control posture.

iii. The Vestibulocerebellum or the flocculonodular lobe of the cerebellum receives substantial amount of inputs from the vestibular nuclei. The Purkinje cells in this part of

the cerebellum do not project to the deep cerebellar nuclei, but directly to the vestibular nuclei. It is involved in controlling balance, vestibular reflexes and eye movements.



Modified and adapted from: http://neuroscience.uth.tmc.edu

Figure 1: Divisions of Cerebellar Cortex: The **posterolateral fissure** separates the **flocculonodular lobe** from the corpus cerebelli, and the primary fissure separates the corpus cerebelli into a **posterior lobe** and an **anterior lobe**. The **vermis** is located along the midsagittal plane of the cerebellum. Lateral side to the vermis is the **intermediate zone** and **lateral hemispheres**.

1.2.2 Cerebellar Cortex histology:

The cerebellar cortex is divided into three layers (See Fig. 2). The innermost layer is the granule cell layer, composed of small, tightly packed granule cells along with interneurons, mostly Golgi cells. The middle layer is the Purkinje cell layer with Purkinje cell bodies and Bergmann glia. The outermost layer; the molecular layer, is made of the axons of granule cells (called parallel fibres), the dendritic arbours of Purkinje cells and inhibitory interneurons, the basket cells and the stellate cells that form GABAergic synapses on to Purkinje cells.

Purkinje cells are the most distinctive neurons in the cerebellum because of their unique and intricate dendritic arbor. They are among the second largest neurons in the brain and were first discovered by the Czech anatomist Jan Evangelista Purkinje in 1837. The apical dendrites of Purkinje cells form a

large fan of finely branched processes. This dendritic arbour is almost two-dimensional, flat and are arranged in a parallel fashion in the molecular layer. The dendrites of Purkinje cells are covered with dendritic spines, each of which receives synaptic input from granule cell parallel fibers. The Purkinje cells integrate and process the motor and sensory information received by parallel fibers (PF) synapses and the inferior olive climbing fiber (CF) synapses. Purkinje cells use GABA as their neurotransmitter and they are at the heart of cerebellar circuitry.

Granule cells in the cerebellum are the most abundant and smallest neurons, estimated roughly 50 billion in human brain. They were first discovered by Camillo Golgi and studied in great detail by Santiago Ramon y Cajal at the end of 19th century. Their cell bodies are packed into the granule cell layer in cerebellar cortex. The dendrites of granules cell are unbranched generally four, short (approx. 13 micrometres in length) innervated by the mossy fibres. Their axon is thin (0.1 micrometre in diameter) and unmyelinated, upsurges through the granule cell layer and then reaches the molecular layer, it bifurcates into two processes to form the parallel fibres in the long axis of the folium. The parallel fibres pass through the dendritic arbours of Purkinje cells, making synaptic connections with Purkinje cell dendritic spines and excite them. Granule cells are the excitatory neurons of the cerebellum and use glutamate as their neurotransmitter. There are two kinds of afferent fibres that transfer impulses to the cerebellar cortex. They are identified on the basis of their morphology and are referred to as mossy and climbing fibres.

Mossy fibres originate in the pontine nuclei, the spinal cord, the brainstem reticular formation, and the vestibular nuclei, and they make excitatory projections onto the cerebellar nuclei and granule cells in the cerebellar cortex. The term mossy fibres was chosen because of the tufted appearance of their synaptic contacts with granule cells. Each mossy fibre innervates hundreds of granule cells. The excitatory neurotransmitter of mossy fibres is glutamate.

Climbing fibres arise exclusively from the inferior olivary nucleus in the brain and make excitatory projections onto the cerebellar nuclei and Purkinje cells of the cerebellar cortex. They are called climbing fibres because their axons climb and wrap around the dendrites of the Purkinje cell like a climbing vine. Each Purkinje cell receives a single, extremely powerful input from a climbing fibre. In contrast to mossy fibres and parallel fibres, each climbing fibre contacts only 10 Purkinje cells on average, making ~300 synapses with each Purkinje cell. Thus, the climbing fibre is a restricted, but extremely powerful, excitatory input onto Purkinje cells. The excitatory neurotransmitter of climbing fibres is probably also glutamate. Both mossy and climbing fibres, which excite their target neurons in the cerebellar cortex, also provide excitatory inputs from collaterals to the deep cerebellar nuclei.

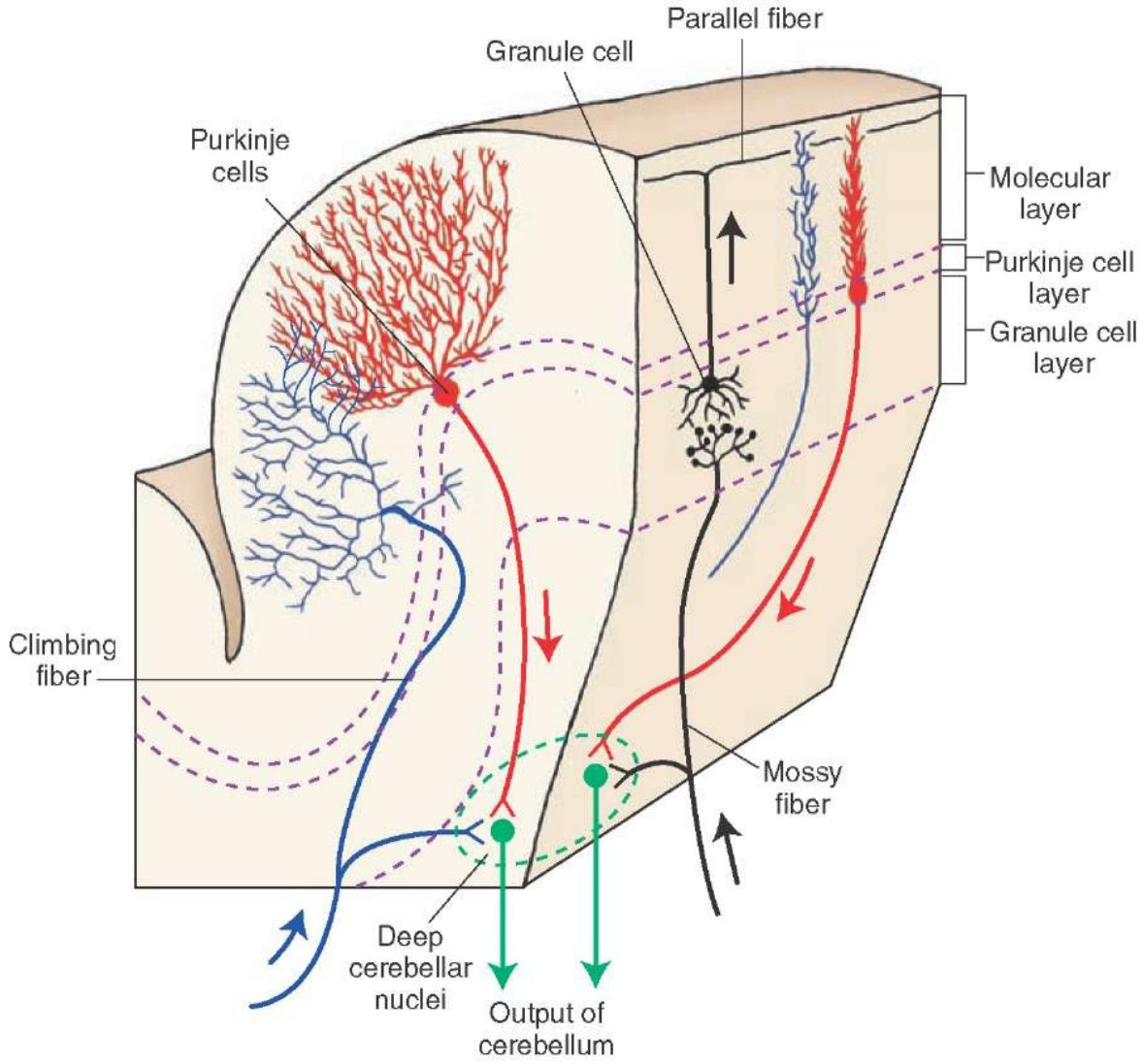
Deep cerebellar nuclei are embedded in the white matter and are known as the dentate, emboliform, globose, and fastigial nuclei. Each pair of deep nuclei is associated with a corresponding region of cerebellar cortex. The dentate nuclei are deep within the lateral hemispheres, the emboliform and globose nuclei (also collectively called interposed nuclei) are located in the intermediate zone, and the fastigial nuclei are in the vermis. Purkinje cell axons enter into the white matter and make inhibitory synaptic connections with the neurons of these nuclei releasing GABA. They also receive glutamatergic excitatory inputs from mossy fibres and climbing fibres. Major output fibres of the cerebellum originate from the deep nuclei except from flocculonodular lobe which synapse on vestibular nuclei directly.

1.2.3. Cerebellar connectivity:

The cerebellar cortex has a relatively simple, stereotyped connectivity pattern that is identical throughout the whole structure (Dean et al., 2010; Reeber et al., 2013). Two main neuronal types, granule and Purkinje cells and four types of interneurons constitute the network in the cerebellar cortex. These neurons are arranged as stereotypic units in a highly precise fashion, each of which is a basic circuit module. The granule cells receive excitatory synapse from mossy fibres arising from neurons in the brainstem or spinal cord and the information from 25 million mossy fibres is passed on to ~50 billion granule cells (Reeber et al., 2013) which is further conveyed to 15 million Purkinje cells via excitatory signals from parallel fibres arising from the granule cells (Fig. 2). Each Purkinje cell receives excitatory signals from a single climbing fibre arising from the inferior olive neurons in the medulla and each climbing fibre innervates to 1–10 Purkinje cells (D'Angelo and Casali, 2012). The initial trace for the memory of a motor sequence is assumed to be stored in the cerebellar cortical circuit and may be consolidated in the deep cerebellar nuclei (Okamoto et al., 2011). This extensive transmission of information from mossy fibres to granule cells and into Purkinje cells is believed to provide a computational benefit for the cerebellar system (Reeber et al., 2013).

1.2.4. Functional and clinical significance:

The cerebellum is a major centre for the integration of sensory and motor information in the brain and plays a vital role in learning and refining motor functions. It contributes to co-ordination, precision and accurate timing in motor activity. The inputs received from the sensory systems of the spinal cord and other parts of the brain are integrated in the cerebellum to synchronize motor activity. Together with the vestibular system, the cerebellum recognizes shifts in balance and maintains the equilibrium.



Adapted from: http://fullthreadahead.com/cerebellum-anatomy-and-function

Figure 2. Mouse Cerebellar circuitry: A schematic illustration of a folium of the mouse cerebellar cortex showing molecular layer; Purkinje cell layer, and granule cell layer. A Purkinje cell and its axon (shown in red) receives excitatory synaptic inputs via granule cell parallel fibres (in molecular layer shown in black) and inferior olive climbing fibres (in blue) and sends inhibitory signals to the neurons of the deep cerebellar nuclei (in green). The granule cell neurons receive excitatory input from mossy fibres which arise from all regions of the brain that project to the cerebellar cortex except inferior olive nucleus.

The cerebellum is also involved in co-ordinated eye movements and believed to have some role in cognitive functions such as thinking, attention, language and mood. It's also thought to be involved in the regulation of fear and pleasure responses (Wolf U. et al., 2009).

Dysfunctions of or damage to the cerebellum can cause either motor or non-motor disorders. Most of the symptoms with cerebellar damage are motor related including dysmetria, hypotonia, tremor, and slurred or dysarthic speech.

The cerebellar ataxias are a group of neurological disorders characterized by gait disturbances, motor incoordination and imbalance, dysarthria, and oculomotor deficits (Klockgether and Paulson, 2011; Manto and Marmolino, 2009). Cerebellar ataxia is the most common form of ataxia. There are more than 60 different forms of inherited cerebellar-based ataxia, with more than half of them classified as either spinocerebellar ataxias, Friedreich's ataxia, episodic ataxia, or fragile X tremor/ataxia syndrome (Durr, 2010; Klockgether, 2010). The Autism Spectrum Disorders are also thought to be linked to cerebellar development and function (Reeber et al., 2013).

There are number of cerebellar malformations which have been reported in humans, primarily based on MRI studies. Most of them also cause cognitive in addition to motor and sensory integration deficits (Bolduc and Limperopoulos, 2009; Tavano et al., 2007). The most common and best understood human cerebellar malformations are listed below:

Dandy Walker Malformation (DWM) is the most common human cerebellar malformation with an estimated incidence of 1/5000 live births (Barkovich et al., 2009 Parisi and Dobyns, 2003). DWM diagnosis is mainly done by imaging and characterized by an enlarged posterior fossa, cerebellar vermis hypoplasia, and an enlarged fourth ventricle. DWM clinical features are variable and patients may show symptoms ranging from intellectual disability to autism (Dobyns et al., 2016).

Joubert syndrome and related disorders (JSRD) is a group of disorders with an incidence of 1 in 80,000–100,000 live births (Kroes et al., 2008; Parisi et al., 2007). JSRD is characterized by cerebellar vermis hypoplasia, thick and abnormally oriented superior cerebellar peduncles, and a deep interpeduncular fossa. Patients with JSRD exhibit variable neurological symptoms such as ataxia, developmental delay, abnormal eye movements, and altered breathing patterns (Dobyns et al., 2016).

Cerebellar hypoplasia refers to the under-development of cerebellum. This cerebellar malformation does not involve a concomitant enlargement of the posterior fossa like DWM. In CH, almost all patients exhibit cognitive and motor impairments (Dobyns et al., 2016).

Cerebellar agenesis is an extremely rare abnormality distinguished by a complete or nearly absence of the cerebellum (Velioglu et al., 1998). Individuals show a number of neurological deficits particularly

related to movement and speech, but can be otherwise surprisingly unaffected (Timmann et al., 2003).

1.2.5. Organotypic cerebellar slice culture

Organotypic slice cultures are a well-established method for culturing tissue slices in which the original cyto-architecture and micro-environment remain intact and accessible. This method has been used in nervous system where most of the neuronal network can be preserved well. Organotypic slice cultures could be referred as "interface" cultures because the slices are lying on the top of a porous membrane which serve as an interface between the air and culture medium (see Fig. 3).

The cellular-architecture of the cerebellum can be preserved well for a longer time in vitro that allows to study cerebellar development, particularly dendritic development of Purkinje cells (Seil FJ, 1972). The interface culture system was first introduced by Stoppini et al., 1991. But it was described in detail by Tanaka et al., 1994 in which they used a membrane floating at the interface between air and medium.

Many researchers use the "cell culture insert" that sits on the top (Tauer et al., 1996; Kapfhammer J.P., 2004) and the slices lying on the membrane can access media from both their apical and basolateral sides. Cerebellar slice cultures are quite different from conventional cell culture because of the thickness and dimension of the tissue.

In cerebellar slice cultures, the cerebellum is sliced in the sagittal plane to preserve the orientation of Purkinje cells in the cerebellar folia. In a 350 µm slice, the Purkinje cells are well-preserved with their dendritic arbour and complete projections to the deep cerebellar nuclei. However, the granule cell parallel fibres are transacted as they traverse perpendicular to the sagittal plane of cerebellum. As the granule cells are still young and immature at the early postnatal stage, the parallel fibres still regrow to make connections with Purkinje cells in cerebellar slice cultures (Kapfhammer J.P., 2005).

Figure. 3

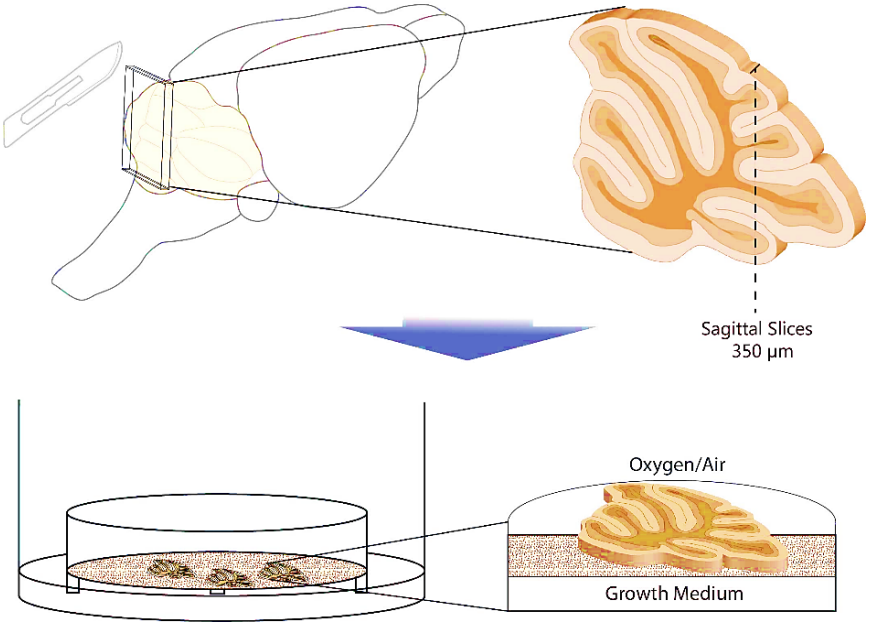


Figure 3: Schematic of Organotypic cerebellar slice culture: The cerebellar slices are laying on to the membrane of tissue culture insert that easily accommodates in to 6-well plate. The membrane is made up of thin mesh with pore size of 1 µm that allows medium and nutrients pass through freely making a thin film around the slices. The cerebellar slices receive sufficient supply of oxygen and nutrients from the medium.

1.3 Purkinje cell dendritic development

1.3.1. Overview

Purkinje cells are the most distinctive neurons in the cerebellar cortex due to their profuse and elaborate dendritic arbour and relatively large soma (approximately, 10-17 µm in diameter at postnatal day 6 and 18 respectively in rats (Takács and Hámori, 1994)). They were first discovered by the Czech anatomist Jan Evangelista Purkinje in 1837. But later in his pioneering work, Santiago Ramon y Cajal revealed the elaborate dendritic arbours of Purkinje cells by using silver impregnation method developed by Camilo Golgi (Ramon y Cajal, S., 1911).

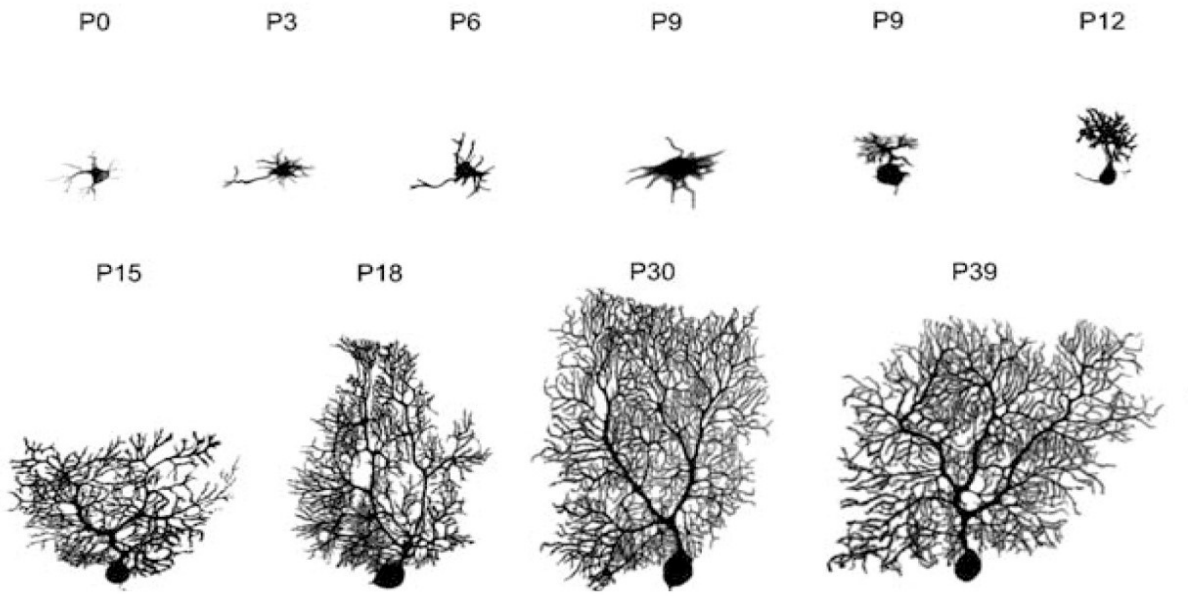
Vast majority of Purkinje cells have a single primary dendrite, but exceptionally some possess two or more which extend towards the molecular layer of the cerebellum, and branch extensively to form secondary or tertiary dendrites. Dendritic spines on tertiary dendrites of Purkinje cells are a major site for synapses with granule cell parallel fibres, which are the most abundant neurons in the brain. Additionally, Purkinje cells are innervated by climbing fibres; axons of inferior olive neurons, and cortical inhibitory interneurons (such as basket and stellate cells) in cerebellar circuitry.

The precise outgrowth and arborisation of dendrites is crucial for their role as integrators of signals relayed from the axon to the corresponding neuron and to fine tune the efficacy of the neurotransmission. Proper dendritic differentiation is particularly resonant for Purkinje cells as the intrinsic activity of this cell-type is governed by functionally distinct regions of its dendritic tree. Activity-dependent mechanisms, driven by electrical signalling and trophic factors, account for the most active period of dendritogenesis.

The development of Purkinje cell dendrites in rat and mouse begins shortly before birth. The dendritic growth is slow in the beginning and little net expansion of the dendritic tree is found up to postnatal day 6 or 7. This period is characterized by the presence of climbing fibre innervation but not parallel fibre innervation (Armengol and Sotelo 1991).

At the end of the first postnatal week parallel fibres appear in the immature molecular layer of the cerebellum, and Purkinje cell dendritic development enters a new phase which is characterized by rapid dendritic expansion and extensive synaptogenesis between Purkinje cell dendrites and parallel fibres (Altman and Anderson, 1972). The dendritic tree of the Purkinje cells in mouse and rat is mature at about 4 weeks of age (see Fig. 4). During this second phase of Purkinje cell dendritic growth, synapses between Purkinje cells and parallel fibres are formed and the Purkinje cell dendritic tree becomes spatially restricted to its plane of orientation. Dendritic arbours expand and become gradually restricted by dendritic remodeling to a single sagittal plane during the third and the fourth week of

postnatal development (Kaneko et al., 2011). It is also during this time that electrophysiological characteristics of Purkinje cells mature (McKay and Turner 2005).



Adapted and modified from McKay and Turner 2005

Fig. 4: Postnatal dendritic development of Purkinje cells in rats starting with P0 to P39 (McKay and Turner 2005).

The development and growth of Purkinje cell dendritic arbor depends on many intrinsic and extrinsic factors, neighboring cell types and tissues, neurotransmitters, hormones and neurotropic factors. Some of them will be discussed in following section.

1.3.2. Cellular and Molecular mechanisms influencing Purkinje cell dendritic development

i. Granule Cells

Cerebellar granule cells are the most abundant neurons in the brain estimated approximately 50 billion in humans and constitute about 1/2 to 3/4 of the total number of neurons in the brain. Cerebellar granule cells are in the granule cell layer of cerebellum, and have an important role for the development of Purkinje cell dendrites. An ablation of granule cells by X-ray irradiation of the brain of Long Evans rats have shown that absence of granule cells and parallel fibres affect the development of Purkinje cell dendritic arbours (Altman and Anderson, 1972). Similar findings have been reported in studies of the mouse mutants weaver and staggerer. In the weaver mutant, very few parallel fibres were

formed, because most of the granule cells degenerated within the external granular layer before they could migrate. In the staggerer mutant, the absence of innervation by parallel fibres resulted in a failure of dendritic spine development on Purkinje cells (Berry et. al., 1978). In dissociated culture, Purkinje cells did not differentiate without granule cells inputs. But in co-culture with granule cells, Purkinje cell differentiation was advanced, resulting in dendrites with dendritic spines (Baptista et. al., 1994). In dissociated cerebellar culture, the activation of NMDA receptors expressed by granule cells triggers the signalling pathway for the dendritic differentiation of Purkinje cells (Hirai and Launey, 2000). These reports confirm that granule–Purkinje cell interactions are crucial for dendritic development of Purkinje cell.

ii. Bergmann Glia

Bergmann glia are unipolar protoplasmic astrocytes in the cerebellar cortex and play an important role in the migration of Purkinje cells and granule cells. Bergmann glial cells have their cell bodies located close to the Purkinje cell layer and extend radial processes enwrapping synapses on Purkinje cell dendrites (Yamada and Watanabe, 2002). During development, Bergmann fibres exhibit a tight association with migrating granule cells. Besides their role in early development of the cerebellum, Bergmann glia are assumed to be required for synaptic pruning (Lippman et al., 2010).

Protein tyrosine phosphatase ζ (PTPζ) is a receptor type protein tyrosine phosphatase which is synthesized as a chondroitin sulfate proteoglycan, and it uses pleiotrophin as a ligand. Pleiotrophin inactivates the phosphatase activity of PTPζ, resulting in the increase of tyrosine phosphorylation levels of its substrates (Fukazawa et al., 2008).

PTPζ is expressed by Purkinje cells and Bergmann glia in the developing cerebellum (Maeda N et al., 1992; Canoll et al., 1993; Matsumoto et al., 1994; Wewetzer et al., 1995; Snyder et al., 1996; Tanaka et al., 2003). The growth of Purkinje cell dendrites occurs throughout the molecular layer and that most dendrites grow in association with radial glia which provide a substrate that directs dendritic growth and might be involved in shaping the Purkinje cell dendritic morphology (Lordkipanidze & Dunaevsky, 2005). In another study, the perturbation of receptor-type protein tyrosine phosphatase (PTN- PTPζ) signalling resulted in a marked increase in the number of Purkinje cells with abnormal dendrites, showing that the Bergmann glia–Purkinje cell interaction is required for the morphogenesis of Purkinje cell dendrites (Tanaka et al., 2003). The morphological aberration of multiple primary dendrites is also found in *ducky* mutant mice which are a model for absence epilepsy characterized by spike-wave seizures and cerebellar ataxia. The *ducky* phenotype is caused by a mutation in Cacna2d2, the gene encoding the α2 delta-2 voltage-dependent calcium channel

accessory subunit. The α2 delta-2 mRNA is strongly expressed in cerebellar Purkinje cells, indicating that pleiotrophin– PTPζ signalling might be associated with voltage-dependent calcium channels that are activated after glutamate stimulation (Brodbeck et al., 2001).

iii. Synaptic activity

In vivo studies have indicated that synaptic activity promotes dendritic arbour elaboration at early stages, and it stabilizes dendritic structure at later stages in the brain development. The different roles of synaptic activity with respect to structural plasticity probably reflect the regulated spatiotemporal expression of key components within signalling pathways (Cline, 2001). The neurotransmitters and neuronal activity regulate both dendritic motility and net dendritic growth (Miller and Kaplan, 2003). The neurotransmission, evoked either spontaneously or by sensory input, triggers changes in intracellular calcium levels that affect the dendritic cytoskeleton. An afferent activity, and the calcium-dependent signalling events mediate the dendritic development (Wong and Ghosh, 2002)**.** In dissociated cerebellar cultures, it has been observed that Purkinje cell dendrites elongate, but as electrical activity emerges the dendrites stop growing, and branch during the first week of cultures (Schilling et al., 1991). But if endogenous electrical activity is blocked by prolonged tetrodotoxin or high magnesium treatment, dendrites continue to elongate, if they were still undeveloped in these cultures. When the dendritic branching begins to develop, the intracellular calcium levels become sensitive to tetrodotoxin treatment, suggesting that this cation might be involved in dendritic development of Purkinje cells (Schilling et al., 1991).

Hormones

iv. Thyroid Hormone

The thyroid hormones, triiodothyronine (T_3) and its prohormone, thyroxine (T_4), are tyrosine-based hormones produced by the thyroid gland that are primarily responsible for the regulation of metabolism. T3 and T_4 are partially composed of iodine. The role of T3 and T_4 hormones have been well assessed in the development of Purkinje cells dendritic arbours. In mouse cerebellar cultures, the addition of a thyroid hormone, T3 or T_4, to the serum-free medium resulted in a highly elaborate dendritic development of Purkinje cells. The cultured Purkinje cells in the presence of T_4 even showed similarities in shape and in synapse formation to normal Purkinje cells in vivo. The effect of T_4 on the dendritic arborisation of Purkinje cells was dose dependent and significantly sensitive to as low as 50 pM. Furthermore, T_4 affected not only Purkinje cell development but also the shape of

other neural cells such as small interneurons (mainly granule cells) and astrocytes in cerebellar cultures (Kimura-Kuroda et al., 2002).

Perinatal T3 deficiency leads to severe cellular perturbations, among them are marked reduction in the growth and branching of Purkinje cell dendritic arborisation. A supplement of triiodothyronine (T_3) or thyroxine (T_4) in dissociated cerebellar cell cultures led to a striking increase in dendritic branching of Purkinje cells in time and dose dependant manner. Triiodothyronine acts on Purkinje cells directly via TRalpha1 expressed in the Purkinje cells, and not in the granule cells, the presynaptic partner of Purkinje cells (Heuer and Mason, 2003).

v. Progesterone and Estradiol

Peripheral steroid hormones secreted by the peripheral steroidogenic glands act on brain tissues via intracellular receptor-mediated mechanisms to regulate several important brain and neuronal functions during development (Tsutsui et al., 2000). These hormones cross the blood–brain barriers, due to their chemical lipid solubility, and induce intracellular receptor-mediated signalling cascade that regulate the transcription of specific genes (Fuxe et al., 1981; McEwen, 1991). Gonadal androgens, for example, act on the brain to influence several reproductive behaviours in vertebrates. Androgenic action in the vertebrate brain is often mediated by the enzymatic activity of cytochrome Aromatase/P_{450}arom which catalyses the conversion of androgen to estrogen. Both P_{450}arom and estrogen receptors are expressed in several brain regions, including the hypothalamus and preoptic area, which are involved in the control of reproductive behaviours. However, more recent findings have suggested that the brain itself synthesize steroids de novo from cholesterol, the so-called neurosteroids (Tsutsui et al., 2000; Compagnone et al., 2000).

In mammals, the Purkinje cell has several kinds of steroidogenic enzymes, such as cytochrome P_{450} side-chain cleavage (P_{450}scc) enzyme, and 3β-hydroxysteroid dehydrogenase/Δ_5- Δ_4-isomerase (3β-HSD), and actively produces progesterone during neonatal life (Furukawa et al., 1998; Ukena et al., 1998, 1999). Apart from being a sex steroid hormone, progesterone also acts on brain tissues through nuclear progesterone receptors (PR) that include the classic nuclear PRA and PRB receptors, and splice variants of each, the seven transmembrane domain 7TMPRβ and the membrane-associated 25-Dx PR (PGRMC1) (Brinton et al., 2008). De novo synthesize of progesterone from cholesterol has been reported in Purkinje cell actively during neonatal life (Sakamoto et al., 2003b), along with 3β-HSD, a membrane-bound mitochondrial enzyme which is involved in biosynthesis of progesterone from pregnenolone (Ukena et al., 1999). Another sex steroid estradiol is thought to act on brain

tissues and in peripheral steroidogenic glands, P_{450}arom is a key enzyme involved in the formation of estrogen.

Tsutsui and colleagues have demonstrated the expression of P_{450}arom in rat Purkinje cells during neonatal life (Sakamoto et al., 2003a). Estradiol promotes dendritic growth, spine formation and synaptogenesis through cognate nuclear receptor in the developing Purkinje cells (Sakamoto et al, 2003c). The knockout mice which lack cytochrome P_{450} aromatase (ArKO), a key enzyme in estradiol synthesis were shown to have reduced dendritic growth, spine formation, and synaptogenesis in Purkinje cells. However, estradiol induces Purkinje dendritic growth, spine formation, and synaptogenesis through BDNF action during development in neonatal wild type mice (Sasahara et al., 2007).

Neurotransmitter

vi. Glutamate

Glutamate is the principal excitatory transmitter in the vertebrate nervous system that acts post-synaptically on three families of ionotropic receptors, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate. In dissociated rat cerebellar cultures, NGF together with the excitatory neurotransmitters such as aspartate or glutamate promoted increase in survival by 2-fold, promoted cell size of Purkinje cells and neurite elaboration (Cohen-Cory et al., 1991). These effects were seen after simultaneous exposure to glutamate or aspartate and NGF or pharmacologic depolarizing agents. Effects on survival or neurite elaboration were not induced by exposure to trophic factors or NGF alone. In cerebellar neuronal cultures, inhibition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropinonic acid (AMPA) or kainate receptor, and metabotropic glutamate receptor (mGluR) leads to the thickening of Purkinje cell dendrites with normal dendrite extension and formation of dendritic spines with substantial reduction of branchpoints (Hirai and Launey, 2000). The effect of NMDA receptor stimulation was indirect and mediated via granule cells, resulting in upsurge of Granule–Purkinje cell interaction, providing neurotrophic elements or electrical activity essential for Purkinje cell differentiation (Hirai and Launey, 2000).

Neurotrophic factor

vii. BDNF

Brain-derived neurotrophic factor, also known as BDNF, is a protein that, in humans, is encoded by the *BDNF* gene. BDNF is a member of the neurotrophin family of growth factors, which are related to the canonical Nerve Growth Factor. BDNF acts on certain neurons of the central nervous system and the peripheral nervous system, helping to support the survival of existing neurons, and encourage the growth and differentiation of new neurons and synapses. BDNF binds at least two receptors on the surface of cells that are capable of responding to this growth factor, TrkB and the p75. Both Purkinje and granule cells in cerebellum express brain-derived neurotrophic factor (BDNF) and the BDNF receptor TrkB (Klein et al., 1993; Yan et al., 1997). In BDNF-knockout mice, increased death of granule cells and reduced Purkinje cell dendritic growth suggested that BDNF is required for normal development and function of the cerebellar cortex (Schwartz et al., 1997). Furthermore, when postsynaptic metabotropic glutamate receptor (mGluR) or inositol 1,4,5-trisphosphate (IP_3) signalling was chronically inhibited in vivo, parallel fibre–Purkinje Cell synaptic strength decreased because of a decreased transmitter release probability. The weakening of synaptic connection caused by the blockade of mGluR–IP_3 signalling was reversed by the in vivo application of BDNF, indicating that a signalling cascade comprising parallel fibre activity, postsynaptic mGluR–IP_3 signalling and subsequent BDNF signalling maintains presynaptic functions in the mature cerebellum (Furutani et al., 2006). Similarly, another study reported that cultured Purkinje cells from inositol 1,4,5-trisphosphate receptor type 1 knock-out (IP_3R1KO) mice exhibited abnormal dendritic morphology. Despite the huge amount of IP_3R1 expression in Purkinje cells, IP_3R1 in granule cells, not in the Purkinje cells, was responsible for the dendritic abnormality of Purkinje cell. However, BDNF application rescued the dendritic phenotype of IP_3R1KO Purkinje cells (Hisatsune et al., 2006).

viii. Corticotropin-releasing factor (CRF) and Urocortin

Corticotropin-releasing factor (CRF), a peptide composed of 41 amino acids, is synthesized in the hypothalamus and regulates the release of adrenocorticotropic hormone from the anterior pituitary (Vale et al. 1981). CRF is present not only in the hypothalamo-pituitary system but also in other regions of the brain (De Souza et al., 1985, 1987; Sakanaka et al., 1987). CRF has been clinically related to the stress axis, depression, anxiety and Alzheimer's disease. However, some studies have shown that it is involved in motor disorders related to the basal ganglia, namely Huntington's

chorea and Parkinson's disease, as well as to olivo-ponto-cerebellar atrophy and spinocerebellar degeneration. In the cerebellum, CRF is concentrated in climbing fibre (CF) afferents, which originate in the inferior olive of the medulla and supply strong excitatory synapses to dendritic spines of the Purkinje cells (Palkovits et al., 1987).

Urocortin; a member of the CRF peptide family has been found to be localized in Purkinje cells axonal terminals, climbing fibres and parallel fibres (Swinny et al., 2002). Both CRF-R1 and CRF-R2 were expressed in climbing fibres from early stages (from P3- to the adult), but CRF-R2 immunoreactivity was only prominent throughout the molecular layer in the posterior cerebellar lobules. (Swinny et al., 2003). In organotypic rat cerebellar cultures, an intermittent exposure of CRF or urocortin (12 hours per day for 10 days ex vivo) induced significantly more dendritic outgrowth (45% and 70%, respectively) and elongation (25% and 15%, respectively) of Purkinje cells when compared with untreated cells. On the contrary, persistent exposure to CRF and urocortin significantly reduced dendritic outgrowth in rat cerebellar cultures (Swinny et al., 2004). Both CRF (CRF-R1 and CRF-R2) and urocortin follow the signal transduction pathway through G-protein coupled receptors and adenylate cyclase that induces the production of the second messenger adenosine 3′,5′-cyclic monophosphate. This study explicitly shows that CRF and urocortin are potent regulators of dendritic development (Swinny et al., 2004).

ix. Homer/Vesl

Homer/Vesl (VASP/Ena-related protein induced during seizure and Long Term Potentiation) proteins localize to the molecular scaffold at postsynaptic densities of excitatory synapses in the mammalian brain (Shiraishi et al.,1999; Kato et al., 2001; Sala et al., 2001; Usui et al., 2003). Postsynaptic targeting of Homer proteins presumably occurs via their binding to proline-rich sequences that are present in type I metabotropic glutamate receptors (mGluRs), inositol (1,4,5)-trisphosphate receptors (IP_3Rs), ryanodine receptors (RyRs) type 1 and 2, C-type transient receptor potential (TRPC) channels, Shank proteins, and dynamin 3 (Ehrengruber et al., 2004). Three isoforms, Homer 1b/c; Homer 2a/b; Homer 3, are reported to be expressed in cerebellum (Xiao et al., 1999). Overexpression of Vesl-1/Homer 1 protein isoforms modulates the ontogenetic development and IP_3- mediated intracellular calcium signalling of rat cerebellar Purkinje cells in organotypic cultures. Morphometric analyses and optical imaging of cytosolic Ca^{2+} transients of cultured Purkinje cells show that the presence of functionally active long isoforms of Vesl-1/Homer 1 critically controls developmental calcium signalling and its effects on dendrite development (Tanaka

et al., 2006). Out of three isoforms, Homer 3a/b is expressed in cerebellum, particularly in the dendritic spines of Purkinje cells and their axons (Shiraishi et al., 2004). Homer3, the predominant isoform in Purkinje cells, is phosphorylated by calcium/calmodulin-dependent protein kinase II (CaMKII) both in vitro and in vivo. The robust phosphorylation of Homer3 and its dissociation from metabotropic glutamate receptor 1alpha (mGluR1alpha) were triggered by depolarization in primary cultured Purkinje cells, and these events were inhibited by CaMKII inhibitor (Mizutani et al., 2008).

x. Ca^{2+}/Calmodulin-dependent protein kinase II

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II or CaMKII) is one of the most prominent protein kinases present in almost every tissue in the brain. CaMKII is a serine/threonine-specific protein kinase that is regulated by the Ca^{2+}/calmodulin complex. Once activated by the binding of calcium/calmodulin, CaMKII switches to a calcium-independent mode in which enzyme activity remains high despite a decrease in cytosolic calcium concentration (Cline, 2001). The substrates phosphorylated by CaMKII are implicated in homeostatic regulation of the cell, as well as in activity-dependent changes in neuronal function including learning and memory (Hudmon and Schulman, 2002).

Neuronal CaM kinase II regulate important neuronal functions, including neurotransmitter synthesis, neurotransmitter release, modulation of ion channel activity, cellular transport, cell morphology and neurite extension, synaptic plasticity, learning and memory, and gene expression (Yamauchi, 2005). Calcium-sensitive enzymes such as CaMKII can influence both neuronal growth and synaptic efficacy. CaMKII is concentrated in postsynaptic densities, with a wide range of substrates including transmitter receptors, channel proteins, and cytoskeletal proteins, it could transduce input activity into coordinated changes in both neuronal growth and synaptic strength (Wu and Cline, 1998). Intracellular calcium signalling mediated by CaMKII and CaMKIV plays an important role in the regulation of dendritic growth during development (Vaillant, 2002).

CaMKII has four isoforms a, b, c and d, of which isoform CaMKIIa is predominantly expressed in the forebrain, while the CaMKIIb isoform is mostly present in the cerebellum (McGuinness et al., 1985; Miller et al., 1985). Brain Ca2+ /calmodulin-dependent protein kinase type II, is a multimeric 600-650 kDa enzyme composed of alpha- (50 kDa) and beta/beta′ (60 and 58 kDa) subunits, the alpha-subunit showed a restricted localization in the rodent cerebellum, particularly in Purkinje cells (Wallas et al., 1988). However, the beta subunit mRNA was expressed in granule cell and Purkinje cells (Burgin et al., 1990).

In dissociated cerebellar cultures, KN62; a CaMKII inhibitor treated cultures showed reduction in number of primary dendrites and the total dendritic length of Purkinje cells. The KN62 affect primary dendrites in between 5 and 15 days in vitro, during which regression and progression of primary dendrites continues actively. On the other hand, AMPA/kainate-type glutamate receptor blockade lead to reduced number of primary dendrites during the same culture period (Tanaka et al., 2006). Another study suggests that elevated levels of Ca^{2+} activate CaMKII, which in turn phosphorylates stathmin, a microtubule destabilizing factor, at Ser16 to stabilize dendritic microtubules. The suppressed stathmin activity by neural activity and CaMKII-dependent phosphorylation at Ser16, leads to dendritic arborisation in dissociated cerebellar cultures (Ohkawa et al., 2007). These findings indicate the importance of neuronal activity and downstream CaMKII signalling in the development of Purkinje cell dendritic arbour.

xi. Retinoid-related orphan receptor RORα and dendritic differentiation

Retinoid-related orphan receptors RORα, −β, and −γ are transcription factors belonging to the steroid hormone receptor superfamily. During embryonic development RORs are expressed in a spatial and temporal manner and are critical in the regulation of cellular differentiation and the development of several tissues. The RORα gene encodes a protein that is a member of a superfamily of nuclear receptors which includes the Peroxisome Proliferator-activated Receptors (PPARs) and the receptors for the glucocorticoids. The RORα gene was identified as the site of mutation in the staggerer mouse which was initially described as ataxic, due to the presence of massive neurodegeneration in the cerebellum.

In ″staggerer″ mutant mice, staggering gait, mild tremor, hypotonia, and small body size are the most prominent features. In these mice, cerebellar cortex is grossly underdeveloped, with a few granule cells and unaligned Purkinje cells (Sidman, et al., 1962). In adult brain, the expression of the RORα gene has been detected in cerebellar Purkinje cells, inferior olive, hippocampus, thalamus and cortex (Matsui et al., 1995). However, some studies have reported that RORα mRNA is highly expressed in the cerebellum and particularly detected in Purkinje cells but not in the granule cell layer (Ino, 2004; Matsui et al., 1995; Nakagawa et al., 1997; Sashihara et al., 1996; Sotelo and Wassef, 1991). RORα plays a key role in dendritic differentiation of Purkinje cells in the cerebellum (Bradley and Berry, 1978; Sotelo, 1990). Purkinje cells change their dendritic morphology in early stage of dendritogenesis. The morphology of Purkinje cells get altered from a fusiform and bipolar shape to a

stellate shape by regression and progression of primitive dendrites and perisomatic protrusions (Ramon y Cajal, 1911; Armengol and Sotelo, 1991).

In mice cerebellar slice cultures, fusiform Purkinje cells with embryonic bipolar shape undergo retraction before the elongation of the dendritic arbour as in vivo. The lentiviral-mediated RORα1 overexpression in fusiform Purkinje cells triggers a cell-autonomous enhanced progression of dendritic differentiation. However, in *staggerer* RORα-deficient mice, Purkinje cells remain in the embryonic fusiform stage and replacement of hRORα1 restores normal dendritogenesis. These findings show that RORα expression in fusiform Purkinje cells is crucial for the dendritic regression and progression of the following step of extension of dendritic processes. However, RORα does not influence in the late dendritic differentiation of Purkinje cell in slice cultures prepared at day 7 (Boukhtouche et al., 2006).

xii. Stathmin Family and dendritic development

Stathmin, also known as Oncoprotein18 (Op18), a ubiquitous cytosoluble phosphoprotein highly expressed in the nervous system. The stathmin family further includes SCG10, SCLIP, RB3, and its splice variants RB39 and RB30, expressed exclusively in various tissues of the nervous system, and possessing a stathmin-like domain (SLD) 1 with various N-terminal extensions. Their involvement in signal transduction and regulation of microtubule dynamics, in relation with their different spatio-temporal expression patterns, suggests that stathmin family proteins may play distinct roles in neuronal activity-dependent dendritic formation (Wong and Ghosh, 2002; Miller and Kaplan, 2003). The regulation of microtubule dynamics is important for the appropriate arborisation of neuronal dendrites during development and has proven critical for the formation of functional neural networks. Op18/stathmin protein family bind tubulin dimers and act by destabilizing microtubules (Cassimeris, 2002; Belmont and Mitchison, 1996). The activities of stathmin are suppressed by phosphorylation by CaMKII at Ser16 which in turn was mediated by the activation of voltage-gated calcium channels and metabotropic glutamate receptor 1. These findings suggest that elevated levels of Ca^{2+} lead to the activation of CaMKII which in turn induce phosphorylation of stathmin to stabilize dendritic microtubules. However, a knockdown of endogenous stathmin by siRNA showed significantly reduced dendritic arbours of Purkinje cells. On the other hand, overexpression of SCG10, a membrane-anchored protein of the stathmin family, restricted the dendritic growth of Purkinje cells (Ohkawa et al., 2007). These findings suggest that stathmin activity is an important regulator in dendritic differentiation of Purkinje cells.

SCLIP is another membrane-anchored protein of the stathmin family (Ozan et al., 1998). Unlike the other stathmins, SCLIP is strongly expressed in Purkinje cells during cerebellar development and accumulates in their dendritic processes at a critical period of their formation and outgrowth (Ozan et al., 1999; Poulain et al., 2008).

Lentiviral-mediated RNAi mediated depletion of SCLIP in cerebellar slice cultures of the embryonic or neonatal cerebellum, promoted retraction of the Purkinje cell primitive process and then prevented the formation of new dendrites at early stages of postnatal development. It also prevented the elongation and branching of dendrites at later phases of differentiation. On the contrary, SCLIP overexpression promoted dendritic branching and development, suggesting that SCLIP is crucial for both the formation and proper development of Purkinje cell dendritic arbours (Poulain et al., 2008). Thus, SCLIP appears to be a novel and specific factor that controls the early phases of Purkinje cell dendritic differentiation during cerebellar development.

xiii. Transcriptional regulation controls dendritic development

Several genes have been identified which play an important role in influencing the late phase of Purkinje cell dendritic development. For example, Flamingo*; a gene in Drosophila* (which is a 7-pass transmembrane cadherin) has shown to play a vital role in patterning dendritic arbour and axon guidance (Shima et al., 2004). A knock down of Celsr2; a mammalian homolog of Flamingo have shown a prominent and simplified dendritic arbours of the Purkinje cells with retraction in dendritic size in organotypic rat cerebellar cultures at P10, suggesting that Celsr2 might be involved in the regulation of growth and maintenance of Purkinje dendrites (Shima et al., 2004).

The Purkinje cell degeneration (pcd) mutant mouse is characterized by mutations in Nna1, a gene that was discovered in the context of axonal regeneration. Its exact function in development and disease is not established yet. Nna1 gene encodes a nuclear protein containing a zinc carboxypeptidase domain. A deletion in exon 7 of Nna1 gene leads to the genetic alteration that causes the spontaneous "Purkinje cell degeneration" (pcd) mutant mouse (Fernandez-Gonzalez et al., 2002). In pcd mutant, Nna1 is responsible for dramatic increase in an intra-nuclear localization of lysyl oxidase pro-peptide, which triggers the deficit of Purkinje cell dendrites by interfering with NF-kB/RelA signalling and microtubule-associated protein regulation of microtubule stability (Li et al., 2010).

In a mouse model of early onset generalized dystonia DYT1 with a loss of function of the torsinA gene the development of the Purkinje cell dendritic tree is impaired. The effect was shown to be mostly cell

autonomous (Zhang et al., 2011). The function of the torsinA gene is not entirely clear but it is an intracellular protein with a chaperone function.

A similar reduction of Purkinje cell dendritic development was found after a loss of function of beta-III spectrin (Gao et al., 2011) highlighting the importance of proper cytoskeletal function for dendritic development. Interestingly, mutations in the beta-III spectrin gene cause spinocerebellar ataxia type 5 with a loss of Purkinje cells (Ikeda et al., 2006, Perkins et al., 2010). Conversely, the ezrin-radixin-moesin (ERM) family member *merlin* which influences the cytoskeleton by interaction with the small GTPases like Ras and Rac appears to inhibit Purkinje cell dendritic growth and expansion (Schulz et al., 2010). Also the remodeling of the extracellular matrix is required for proper development of Purkinje cell dendritic arbour. Purkinje cell dendritic growth was impaired in mice deficient with the matrix metalloproteinase-3 and the final dendritic trees of the Purkinje cells were reduced in size (Van Hove et al., 2012).

2. CALCIUM SIGNALLING AND PURKINJE CELL DENDRITIC DEVELOPMENT

2.1. Ca^{2+} as a signalling molecule

Many hormones, neurotransmitters and other signalling molecules direct cellular function via membrane receptors that are coupled to second messengers. Second messengers, in turn, activate enzymes that coordinate cellular responses to extracellular stimuli (Carafoli and Klee, 1999). Calcium (Ca2+) has proven to be a universal second messenger in eukaryotic cells that accumulates in the cytoplasm in response to diverse classes of stimuli and regulates many aspects of cell function. In neurons, Ca2+ influx in response to action potentials or synaptic stimulation triggers neurotransmitter release, modulates ion channels, induces synaptic plasticity, and activates transcription (Higley and Sabatini, 2012).

Ca^{2+} plays an essential role in vesicle fusion and is therefore central to the basic operation of neurons through synaptic communication. It is also essential in muscle physiology, where it couples membrane excitation with contraction. Additional Ca^{2+}-regulated intracellular responses include glycogenolysis, mitochondrial respiration, endocytosis, and neurotransmitter synthesis (Carafoli and Klee, 1999).

In general, cells maintain an intracellular Ca^{2+} level of 10^{-7}M, which is 10^{-4} times lower than the level outside the cell, by sequestering Ca^{2+} in several intracellular organelles. An increase in intracellular Ca^{2+} (up to 10^{-4}M) may be derived from extracellular and intracellular sources in response to agonists and membrane-depolarizing stimuli.

Purkinje cells possess multiple mechanisms to modulate ($[Ca^{2+}]i$), for example Ca^{2+}-binding proteins, intracellular Ca^{2+} stores, plasma membrane Ca^{2+}-ATPase, Na^{+}/Ca^{2+} exchanger (NCX), P/Q-type and T-type Ca^{2+} channels. A few mechanisms that influence the dendritic development of Purkinje cells are explained in following sections:

2.1.1. *Voltage-gated calcium channels (VGCC)*

Voltage-gated calcium channels are protein complexes that mediate calcium influx in response to membrane depolarization. High threshold VGCC (L-type, P/Q-type, N-type and R-type) are activated by strong depolarization, whereas low threshold calcium channels (T-type) open in response to mild depolarization steps (Nimmrich and Gross, 2012). The diversity of Ca2+ channels in vertebrate neurons is of fundamental interest because voltage-gated Ca2+ entry through these channels controls a wide variety of physiological functions, encompassing neurotransmitter release,

membrane excitability, neurite outgrowth, bioenergetics, and gene expression (Reuter, 1983; Hille, 1992).

The P/Q-type calcium channel (also referred to as $Ca_v2.1$) is a presynaptic high-voltage-gated calcium channel, which couples neuronal excitation to secretion of neurotransmitter (Ishikawa et al., 2005). P-type channels were first identified in Purkinje neurons of the cerebellum (Llinás et al., 1989) and were distinguished from Q-type channels identified in cerebellar granule neurons (Randall and Tsien, 1995). Both P/Q-type channels are characterized by their sensitivity to the venom of *Agelenopsis aperta*, ω-agatoxin IVA (Mintz et al., 1992a).

T-type voltage-gated calcium (Ca^{2+}) channels are expressed in a wide range of tissues, including the nervous, cardiovascular, and endocrine systems (Perez-Reyes, 2003). The T-type Ca^{2+} channel family is composed of three main subtypes; $Ca_v3.1/\alpha_{1G}$, $Ca_v3.2/\alpha_{1H}$, and $Ca_v3.3/\alpha_{1I}$ (Talley et al., 1999; McKay et al., 2006) and are functionally expressed in the nervous system, particularly in dendritic spines (Carter and Sabatini, 2004; Isope and Murphy, 2005). Out of the three subtypes, Ca_v 3.1 and Ca_v 3.3 T-type Ca^{2+} channel isoforms are expressed in cerebellar Purkinje cells (Talley et al., 1999; Molineux et al., 2006) and T-type channels are preferentially expressed in Purkinje cell dendritic spines and colocalize with mGluR1s (Hildebrand et al., 2009).

T-type and P / Q-type channels are abundantly expressed in Purkinje cell dendrites (Usowicz et al., 1992; Hildebrand et al., 2009), and constitute one of the major sources of Ca^{2+} influx into Purkinje cells (Usowicz et al., 1992; Watanabe et al., 1998; Isope & Murphy, 2005; Isope et al., 2012). Inhibition of P/Q and T-type Ca^{2+} channels alone in cerebellar slice cultures does not affect dendritic arbour development of Purkinje cells, but it rescues the dendritic reduction caused by activation of mGluR1 by DHPG (Gugger et al., 2012).

2.1.2. *Metabotropic glutamate receptors (mGluRs)*

The metabotropic glutamate receptors (mGluRs) are the members of the G-protein-coupled receptor (GPCR) superfamily, the most abundant receptor gene family in the human genome. Similar to glutamate receptors, mGluRs bind glutamate, an amino acid that functions as an excitatory neurotransmitter. There are eight different types of mGluRs; mGluR1- mGluR8 and they are divided into groups I, II and III based on receptor structure and physiological action. The members of mGluRs in group I, are mGluR1 and mGluR5, and they are activated strongly by the orthosteric agonist (S)-3,5-Dihydroxyphenylglycine or DHPG. In general, group I mGluRs couple with Gq/G11 and activate phospholipase Cβ, resulting in the hydrolysis of phosphotinositides and generation of

inositol 1,4,5-trisphosphate (IP_3) and diacyl- glycerol. This classical pathway leads to Ca2+ mobilization and activation of protein kinase C.

In cerebellar neuronal cultures, endogenous activation of mGlu1 and mGlu5 receptors contributes to cerebellar development and selective blockade of these receptors differentially affects the maturation of Purkinje cells (Catania et al., 2001). On the contrary, the suppression of glutamate mediated neurotransmission did not change the development of Purkinje cell dendrites and they appeared normal in cerebellar slice cultures (Adcock et al., 2004).

Chronic stimulation of the metabotropic glutamate receptor 1 with DHPG caused a severe dendritic reduction of Purkinje cells in mice cerebellar slice cultures (Sirzen-Zelenskaya et al., 2006; Gugger et al., 2012), but this dendritic reduction was partially rescued after inactivation of P/Q and T-type Ca^{2+} channel (Gugger et al., 2012).

2.1.3 *Protein Kinase C*

Protein kinase C (PKC) refers to a large family of protein kinase enzymes that are involved in regulating the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues. PKC enzymes in turn are activated by signals such as increases in the concentration of diacylglycerol (DAG) or calcium ions (Ca2+). Hence, PKC enzymes play important roles in several signal transduction cascades.

Protein kinase C (PKC) is one of the important molecules in signal transduction for the expression of Long Term Depression (LTD) at the parallel fibre–Purkinje cell synapse (Ito M., 2001). Purkinje cell dendritic development depends on the activity of Protein kinase C (Metzger and Kapfhammer, 2000). In PKCγ-deficient mice, Purkinje cell dendritic arbours were expanded and having an increased number of branchpoints compared to control mice indicating a role of PKCγ isoform as a negative regulator of dendritic growth and branching. The branching-stimulating phenotypes of the PKC inhibitors 2-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl) maleimide and Go«6976 found in wild-type cultures were absent in PKCγ deficient mice (Schrenk et al., 2002).

2.1.4. *Plasma membrane calcium ATPase 2 (PMCA2)*

The Plasma membrane calcium ATPase isoform 2 (PMCA2) is one of four mammalian isoforms of the PMCA family. The PMCAs are calcium-transporting P-type ATPases responsible for the extrusion of ionized calcium (Ca^{2+}) from the cytosol to the extracellular environment. The PMCAs form an obligatory aspartyl-phosphate intermediate during the reaction cycle (hence their classification as P-

type ATPases). These antiporters are responsible for the resetting and maintenance of resting levels of intracellular free Ca^{2+} and may be involved in local and dynamic regulation of Ca^{2+} signalling in diverse tissues and cell types. The PMCAs generally exhibit low activity at (50 nM) Ca^{2+} concentrations and are activated by interaction with Ca^{2+}-calmodulin.

PMCA2 has a high basal activity and is one of the fastest pumps with respect to Ca^{2+} -calmodulin activation. PMCA2 knockout (PMCA2-/-) mice have an overt "cerebellar" ataxia phenotype. These mice are deaf and have vestibular abnormalities (Kozel et al., 1998). PMCA2 is the dominant calcium transporter, highly expressed throughout the soma, dendrites and dendritic spines of the Purkinje cells (Filoteo et al., 1997; Hillman et al., 1996; Sherkhane and Kapfhammer, 2013). The expression of PMCA2 in Purkinje cell dendrites and dendritic spines has been studied and its role in dendritic development of Purkinje cell will be discussed in chapter 3.

2.1.5. *Na^{+}/Ca^{2+} exchanger (NCX)*

Among the different pathways that mediate Ca^{2+} ions, the Na^{+}/ Ca^{2+} exchanger has emerged as a predominant mechanism for Ca^{2+} efflux across the plasma membrane, particularly when overall Ca^{2+} levels in cells are elevated (Lee et al., 2002; Kim et al., 2005; Wanaverbecq et al., 2003). The Na^{+}/Ca^{2+} exchange was first described in the squid axon and mammalian heart by P. F. Baker and H Reuter in 1968. Several studies have demonstrated that Na^{+}/Ca^{2+} exchanger plays a vital role in Ca^{2+} extrusion and operates with a stoichiometry of three Na^{+} ions to one Ca^{2+} ion (3:1), although this ratio varies according to the intracellular ion concentration (Blaustein and Lederer, 1999). The Na^{+}/Ca^{2+} exchanger (NCX) is responsible for most of the efflux of Ca^{2+} from the cell. The NCX transporter is a member of the SLC8 family of solute carriers which in turn belong to the CaCA superfamily (Philipson and Nicoll, 2000; Lytton, 2007; On et al., 2008). NCX has three isoforms; NCX1, NCX2 and NCX3. NCX1 is ubiquitously expressed in most of the mammalian cells, its expression is detected particularly in the heart, brain, and kidney. NCX2 expression is much more limited; it is expressed only in neurons. NCX3 is expressed in skeletal muscle and in some regions of the brain (Lytton, 2007). Due to their central role in modulating Ca^{2+} levels in the cell, Na^{+}/Ca^{2+} exchangers are involved in various pathophysiological conditions such as hypoxia, aging and Alzheimer's disease. Expression of Na^{+}/Ca^{2+} exchanger isoforms in mouse cerebellum and their role in dendritic development of Purkinje cells will be described in chapter 4.

3. SPECIFIC AIMS OF THE THESIS

3.1 The role of Plasma membrane calcium ATPase 2 (PMCA2) in Purkinje cells dendritic development

This project was a first step to understand the role of calcium clearance mechanisms in the development of Purkinje cell dendritic arbor in mouse cerebellar slice cultures. The major Ca^{2+} extrusion mechanisms in Purkinje cells are the Plasma membrane calcium ATPase 2 (PMCA2) and Na^{+}/Ca^{2+} exchanger (NCX). These two antiporters deal with the Ca^{2+} homeostasis in Purkinje cell dendrites. In this thesis, I addressed the question: What is the role of Ca^{2+} clearance mechanisms in Purkinje cells dendritic development?

Previous research in our lab suggests that metabotropic glutamate receptors type I (mGluR1s) and protein kinase C (PKC) play a key role in growth and development of Purkinje cell dendritic arbors. Chronic activation of mGluR1 by DHPG and PKC by PMA has detrimental effect on the development of Purkinje cell dendritic arbor (Metzger and Kapfhammer, 2000, Schrenk et. al., 2002, Sirzen-Zelenskaya et al., 2006). This effect could be partially rescued by pharmacological blockade of P/Q and T-type Ca^{2+} channels, indicating that activation of these channels leads to Ca^{2+} influx in the cells and contributes to the reduction of dendritic growth (Gugger et. al., 2012). Besides the influx of Ca^{2+} through voltage-gated ion channels, calcium clearance mechanism also affects the calcium equilibrium in Purkinje cells. The plasma membrane Ca^{2+}-ATPase2 (PMCA2) is reported to be involved in extrusion of Ca2+ and cerebellar synapse function (Huang et. al., 2010).

We decided to test the role of PMCA2 for the development of Purkinje cell dendritic arbor. In chronic activation of mGluR1 or PKC, the dendritic arbor size of Purkinje cells is severely compromised and we wanted to test whether PMCA2 was involved in this mechanism. We chronically treated mice cerebellar slice cultures with CEDA-SE (5-(and-6)-Carboxyeosin diacetate Succinimitidyl Ester, a PMCA2 inhibitor, and we found that there was a slight reduction of Purkinje cell dendritic arbor size compared to control. But, when we co-treated the cultures with (RS)-3,5-Dihydroxyphenylglycine, we could see the reduced dendritic arbor was partially rescued. A likely explanation is that PMCA2 inhibition leads to compensatory inactivation of P/Q-type and T-type Ca^{2+} channels which were presumably activated by the stimulation of mGluR1.

3.2 The role of Na^{+}/Ca^{2+} exchanger (NCX) in Purkinje cells dendritic development

The aim of this project was to test the role of Na^{+}/Ca^{2+} exchanger in dendritic development of Purkinje cells. The Na^{+}/Ca^{2+} exchanger, referred shortly as NCX is another Ca^{2+} exchange mechanism in Purkinje cells that mediates Ca^{2+} and Na^{+} fluxes across the synaptic plasma membrane.

This transporter is so called bi-directional, with two modes of operation: the forward mode which pumps Ca^{2+} out of the cell and the reverse mode that allows Ca^{2+} to enter in the cell (Blaustein MP and Lederer WJ., 1999; Philipson KD and Nicoll DA., 2000; Tong and Hilgemann, 2004). NCX, together with selective ion channels and ATP-dependent pumps, controls the physiological cytosolic concentrations of Na^{+} and Ca^{2+} ions (Blaustein MP and Lederer WJ., 1999). It was also reported that NCX may play an important role in modulating neuronal activity and affecting excitotoxicity (Storozhevykh et al., 1998; Thayer et al., 2002). NCX-mediated Ca^{2+} influx and removal of intracellular Ca^{2+} can modulate synaptic transmission (Scotti et. al., 1999). We decided to test whether interfering with Na^{+}/Ca^{2+} exchanger by pharmacological inhibitors would affect Purkinje cell dendritic development in cerebellar slice cultures.

The blockade of the forward mode of NCX (Ca^{2+} efflux mode) by Bepridil inhibited the growth and development of the Purkinje cell dendritic arbor moderately. However, the blockade of the reverse mode (Ca^{2+} influx mode) by KB-R7943 severely reduced the development of the dendritic arbor and yielded Purkinje cells with thickened distal dendrites. Further, we tested whether this phenotype was apparent in the absence of bioelectrical activity and whether it was mediated by the activity of voltage-gated calcium channels.

Initially, we hypothesized that the thickened distal dendritic phenotype is unrelated to NCX function. We used additional NCX pharmacological inhibitors like CB-DMB, ORM-10103, SEA0400, YM-244769 and SN-6 which have higher specificity for NCX isoforms in terms of their target preference, either for the forward, reverse or both modes. All of these inhibitors caused a strong dendritic reduction without producing the thickened distal dendrites.

Our findings indicate that Na^{+}/Ca^{2+} exchanger is an important regulator of the intracellular calcium equilibrium and disturbance with its function could affect the Purkinje cells dendritic development. These finding also emphasize the importance of the calcium equilibrium or homeostasis for the normal dendritic development and function of cerebellar Purkinje cells.

The Plasma Membrane Ca^{2+}-ATPase2 (PMCA2) is involved in the regulation of Purkinje cell dendritic growth in cerebellar organotypic slice cultures

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4.1 Abstract

Purkinje cells are the principal neurons of the cerebellar cortex and have an extensive and elaborate dendritic tree. Chronic activation of type I metabotropic glutamate receptors inhibits Purkinje cell dendritic growth in organotypic cerebellar slice cultures. This effect is mediated by calcium influx through P/Q-type and T-type Ca^{2+} channels. We have now studied the role of the plasma membrane Ca^{2+}-ATPase2 (PMCA2), a major calcium extrusion pump, for Purkinje cell dendritic development. We found that PMCA2 is strongly expressed in the plasma membrane and dendritic spines of Purkinje cells in organotypic slice cultures compatible with a role for controlling the local dendritic calcium equilibrium. Inhibition of PMCA2 activity by carboxyeosin resulted in a moderate reduction of Purkinje cell dendritic tree size indicating that the extrusion of calcium by PMCA2 is important for maintaining the dendritic calcium concentration and for controlling dendritic growth. When inhibition of PMCA2 was combined with stimulation of type I metabotropic glutamate receptors, it partially rescued dendritic morphology. This protection can be explained by a compensatory inactivation of voltage-gated calcium channels in Purkinje cells after PMCA2 inhibition. Our results demonstrate that PMCA2 activity is an important regulator of the dendritic calcium equilibrium controlling Purkinje cell dendritic growth.

4.2. Introduction

Purkinje cells are the principal neurons of the cerebellar cortex and have an extensive and elaborate dendritic tree. They receive excitatory synaptic input from granule cell derived parallel fibers and inferior olive derived climbing fibers. The development of the Purkinje cell dendritic tree is controlled by a variety of intrinsic and extrinsic signals (Kapfhammer JP, 2000; Kazuto Fujishima et al., 2012). We have previously shown that chronic activation of either type I metabotropic glutamate receptors (mGluR1s) or protein kinase C (PKC) in organotypic cerebellar slice cultures severely inhibits the growth and development of the Purkinje cell dendrites (Metzger and Kapfhammer, 2000; Shrenk et al., 2002; Sirzen-Zelenskaya et al., 2006). The stunted dendritic growth seen after mGluR1 or PKC stimulation is partially rescued by pharmacological blockade of P/Q-type and T-type Ca^{2+} channels, indicating that activation of these channels mediating Ca^{2+} influx contributes to the inhibition of Purkinje cell dendritic growth [Gugger et. al., 2012]. Besides the influx of calcium through voltage-dependent channels, calcium clearance mechanisms also affect the calcium equilibrium in Purkinje cells (Huang et al., 2010, Kim et al., 2007, Roome et al., 2013). The plasma membrane Ca^{2+}-ATPase2 (PMCA2) is reported to be involved in extrusion of calcium and cerebellar

synapse function (Huang et al., 2010). PMCA2 belongs to the family of P-type primary ion transport ATPases characterized by the formation of Aspartyl phosphate intermediate during an ATP hydrolysis reaction cycle. Of the known PMCA variants, PMCA1 and PMCA4 are expressed ubiquitously whereas PMCA2 and PMCA3 are expressed prevalently in the central nervous systems. The PMCA2 isoform is highly expressed in the cerebellum, particularly in Purkinje cell dendrites and dendritic spines (Filoteo et al., 1997, Hilman et al., 1996). Two spontaneous mouse mutants with a loss of function of PMCA2 (Street et. al., 1998 Uno et. al., 2002) and a PMCA2 knockout mouse (Kazel et al., 1998) are known. They are characterized by a combination of deafness with a marked cerebellar ataxia. The aim of this study was to investigate whether PMCA2 activity may be involved in Purkinje cell dendritic growth and whether it would be modulating the effects of mGluR1 activation on the development of the Purkinje cell dendritic tree. PMCA2 can be pharmacologically inhibited by treatment with carboxyeosin diacetate succinimidyl ester, shortly known as carboxyeosin. We have studied the effect of inhibiting PMCA2 by carboxyeosin on Purkinje cell dendritic growth and tested whether carboxyeosin treatment might modulate the reduction of the Purkinje cell dendritic tree seen after mGluR1 activation.

4.3. Materials and Methods

4.3.1. *Organotypic slice cultures*

Animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and permitted by Swiss authorities. Cultures were prepared from B6CF1 mice (CB6) as described previously (Adcock et al., 2004, Kapfhammer, 2005, Kapfhammer & Gugger, 2012). Mouse pups were decapitated at postnatal day 8 and their brains were dissected aseptically. The cerebellum was separated in ice-cold preparation medium (minimal essential medium (MEM), 1% glutamax (Gibco, Invitrogen), pH 7.3) and slices of 350 µm thickness were cut with a McIllwain tissue chopper under sterile conditions. Cerebellar slices were separated, transferred on to a permeable membrane (Millicell-CM, Millipore) and incubated with incubation medium (50% MEM, 25% Basal Medium Eagle, 25% horse serum, 1% glutamax, 0.65% glucose) with 5% CO_2 at 37°C. The medium was refreshed every 2-3 days. The following pharmacological compounds were added to the medium at each change for a total of 7 days, starting at 2-4 days in vitro (DIV): (RS)-3,5-Dihydroxyphenylglycine (DHPG, Tocris, Bristol, United Kingdom), carboxyeosin diacetate succinimidyl ester (Carboxyeosin, Molecular Probes, USA). The following concentrations were used: 10 µM DHPG, 10 µM and 20 µM carboxyeosin. When

carboxyeosin was used in combination with DHPG it was added 24h prior to the first DHPG treatment. Slices were kept in culture for a total of 9-11 days before fixation and immunohistochemical staining.

4.3.2 *Immunohistochemistry*

At DIV 9-11 cultures were fixed in 4% paraformaldehyde for 6-24h hours at 4°C. All reagents were diluted in 100 mM phosphate buffer (PB), pH 7.3. Antibodies were added to the slices in fresh blocking solution (PB + 3% non-immune goat serum + 0.3% Triton X-100) and incubated overnight at 4°C. After washing in PB, secondary antibodies were added to the slices in PB containing 0.1% Triton X-100 for 2 hours at room temperature. For the analysis of Purkinje cell dendritic size, rabbit anti-Calbindin D-28K (Swant, Marly, Switzerland, 1:1000) was used as a primary antibody and goat anti-rabbit Alexa 568 (Molecular Probes, Invitrogen, 1:500) was used as a secondary antibody. To validate the expression of PMCA2 in Purkinje cell dendrites and dendritic spines, rabbit anti-Calcium Pump PMCA2 ATPase antibody (Abcam, United Kingdom, 1:500) was used as a primary antibody and goat anti-rabbit Alexa 488 (Molecular Probes, Invitrogen, 1:500) was used as a secondary antibody. For double-staining with calbindin a mouse monoclonal anti-Calbindin D-28K antibody (Swant, Marly, Switzerland) was used in combination with the PMCA2 antibody. Stained slices were mounted on glass slides with coverslip using Mowiol. The microscopic observations were made on an Olympus AX-70 microscope equipped with a Spot Insight digital camera. Images were processed for optimization of brightness and contrast with Adobe Photoshop software.

4.3.3. *Quantitative analysis of cultured Purkinje cell dendrites*

The quantification of Purkinje cell dendritic tree size was done as previously described (Adcock *et al.*, 2004; Kapfhammer, 2005). Purkinje cells which had a dendritic tree not overlapping with neighbouring cells were selected for analysis. An image analysis program (Image Pro Plus) was used to trace the outline of the Purkinje cell dendritic tree yielding the area covered by the dendritic tree. Purkinje cells were acquired from three independent experiments with an average number of 20 cells per experiment and per growth condition. For detailed information on the number of cells measured for each diagram see supporting Table 1 & 2. The data were analyzed using GraphPad Prism software. The mean value of the dendritic tree area of untreated control cells were set to 100 % and the results were expressed as percentage of controls. Error bars represent the standard error of the mean (SEM). The statistical significance of differences in parameters was assessed by non-parametric analysis of variance (Kruskal-Wallis test) followed by Dunn's post test. For comparisons of single data columns,

Mann-Whitney's non-parametric test was used. Confidence intervals were 95%, statistical significance was assumed with $p<0.05$.

4.4. Results

4.4.1. *The Plasma membrane Ca^{2+} ATPase PMCA2 is strongly expressed in Purkinje cell dendrites in cerebellar slice cultures*

T- and P/Q-type Ca^{2+} channels are abundantly expressed in Purkinje cell dendrites (Usowicz et al., 1992, Hildebrand et al., 2009) and are one of the major sources of Ca^{2+} influx into Purkinje cells (Usowicz et al., 1992, Watanabe et al., 1998, Isope & Murphy, 2005, Isope et. al., 2012). Furthermore, Ca^{2+} influx through these channels has been shown to be potentiated by mGluR1 activation (Hildebrand et al., 2009, Kitano et al., 2003, Johnston & Delaney, 2010). The plasma membrane Ca^{2+} ATPase (PMCA2) is abundantly and highly expressed in the Purkinje cell dendrites and dendritic spines (Filoteo et al., 1997, Hilman et al., 1996) and plays a crucial role in calcium dynamics and synaptic communication at cerebellar synapses (Huang et al., 2010).

In order to verify the expression of PMCA2 independently in Purkinje cell dendrites and dendritic spines in cerebellar slice cultures, we performed immunohistochemistry with an anti-PMCA2 antibody on cerebellar slices after 10 DIV. We found that PMCA2 immunoreactivity (IR) was strongly present on the dendritic tree of the Purkinje cells, while there was only little expression on the axon, the cell soma and the stem dendrites (Fig. 5 A). This became further evident in double-stainings with anti-calbindin which stained all parts of the Purkinje cells with a similar intensity. Therefore, the cell soma and proximal dendrites appear red in the double staining, whereas the distal dendritic tree appears yellow to green due to the strong presence of PMCA2 IR. (Fig. 5 B) When the distal dendrites were viewed in high magnification it could be seen that PMCA2 was strongly expressed at the dendritic plasma membrane and in dendritic spines of Purkinje cells (arrows in Fig 5 C). When cerebellar slice cultures were treated chronically with the PMCA2 inhibitor carboxyeosin for 7 days, there was no major change in the expression pattern of PMCA2 in the Purkinje cell dendritic tree. PMCA2 staining remained strong and was concentrated in the plasma membrane of the peripheral part of the Purkinje cell dendritic tree (Fig. 5, D – F) indicating that chronic inhibition of PMCA function did not affect the expression or cellular distribution of PMCA2 in a major way.

4.4.2. *Chronic inhibition of PMCA2 by carboxyeosin induced a moderate reduction of Purkinje cell dendritic tree size*

In studies with PMCA2 knockout mice, it was shown that Purkinje cells in these mice have a reduced size with a stunted dendritic tree (Empson et al., 2007) and have an increased resting calcium level

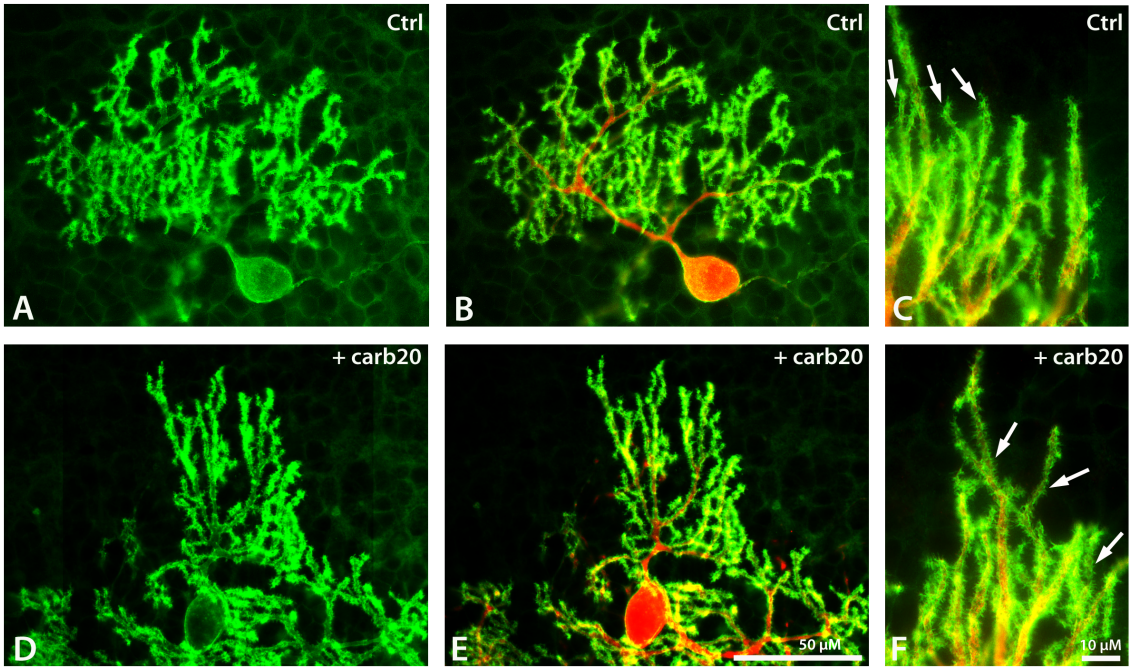

Fig. 5: Staining of anti-calcium pump PMCA2 ATPase and Calbindin D-28K in Purkinje cells in organotypic slice cultures: (A-C) untreated control cultures (D-F) Caboxyeosin-treated cultures. A) Untreated control showing abundant expression of PMCA2 in dendrites and dendritic spines (green) B) Merged red Purkinje cell soma with higher expression of PMCA2 in dendrites. C). Abundant expression of PMCA2 in dendritic plasma membrane and dendritic spines. D) Carboxyeosin treated Purkinje cell showing abundant expression of PMCA2 in dendrites and dendritic spines (green) E) Red Purkinje cell soma with higher expression of PMCA2 in dendrites and dendritic spines. F) Abundant expression of PMCA2 in dendritic plasma membrane and dendritic spines. Scale bar 50 µm (A-B, D-E) and 10 µm (C-F).

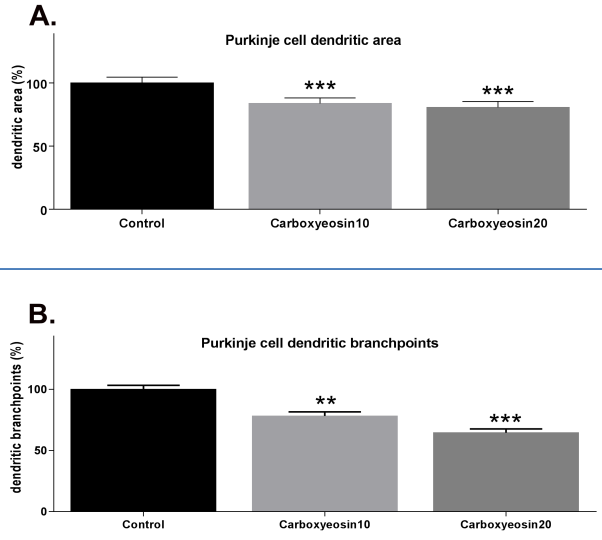

Fig. 6: A) - Size of Purkinje cell dendritic arbors in control and carboxyeosin-treated cultures: The mean size of control Purkinje cells was considered as 100%. Purkinje cell dendritic trees from carboxyeosin-treated cultures were significantly smaller compared to control cultures with $p<0.05$ (*) for 10 mM carboxyeosin and $p<0.01$ (**) for treatment with 20 mM carboxyeosin. **B) - Number of branch points with carboxyeosin:** The mean number of branch points of control Purkinje cells was considered as 100%. Carboxyeosin treatment alone showed a reduction of the number of branch points to 73% with 10 µM and 62% with 20 µM compared to control values. These differences were significant with $p<0.01$ (**) for 10 mM carboxyeosin and with $p<0.001$ (***) for 20 µM carboxyeosin. Error bars represent the SEM.

(Empson et al., 2010). We tested whether the chronic functional inhibition of PMCA2 in cerebellar slice cultures would also affect the development of the Purkinje cell dendritic tree.

After 7 days carboxyeosin treatment, we found a moderate but significant reduction of the Purkinje cell dendritic tree size by about 20% both for treatment with 10 µM and 20 µM carboxyeosin (Fig. 6, Fig. 7C, supplemental table 1).

This finding is in line with the observations in PMCA2 knockout mice (Empson et al., 2007) and shows that chronic carboxyeosin treatment mimics a loss of function of PMCA2 and that dendritic growth under these conditions is reduced. Because PMCA2 is an important regulator of the calcium homeostasis in Purkinje cell dendrites, we studied whether the mGluR1 effect might be mediated by PMCA2. We pretreated cerebellar slice cultures with the PMCA2 inhibitor carboxyeosin for 1 day followed by co-application of DHPG and carboxyeosin for 7 days.

We found that pretreatment with carboxyeosin had a strong rescuing effect for the Purkinje cell dendritic tree after mGluR1 activation by DHPG treatment (Fig. 7 D). The dendritic tree of Purkinje cells from co-treated slice cultures appeared similar to that after carboxyeosin treatment alone (Fig. 7 C, Fig. 8), but was much larger than after DHPG treatment alone (Fig. 7 B, Fig. 8). Qualitatively, there was a reappearance of the peripheral Purkinje cell dendritic branches after co-treatment and the overall shape of the dendritic tree resembled that of cells from carboxyeosin treatment alone (Fig. 7 C).

The quantitative data indicate that mGluR1 stimulation with DHPG gives rise to a reduction of the size of dendritic arbors to 48% of the size in untreated control cultures. In contrast, co-treatment with carboxyeosin resulted in a rescue of the dendritic area to 70% of that in untreated control cultures, i.e. dendritic tree size was increased by 45% compared to DHPG treatment alone (Fig. 8, supplemental table 1). In fact, the size of Purkinje cell dendritic tree co-treated with carboxyeosin and the mGluR1 agonist DHPG was similar to those treated with carboxyeosin alone indicating that the mGluR1 activation resulted in no additional inhibition of dendritic growth. These differences were significant with $p < 0.001$ confirming the rescuing effect of carboxyeosin treatment for mGluR1-mediated dendritic reduction.

4.5. Discussion

The major finding of this study is that PMCA2 inhibition with carboxyeosin by itself was reducing the size of the Purkinje cell dendritic tree, but at the same time it had a strong rescuing effect for the dendritic tree when combined with mGluR1 stimulation through DHPG. The finding that PMCA2

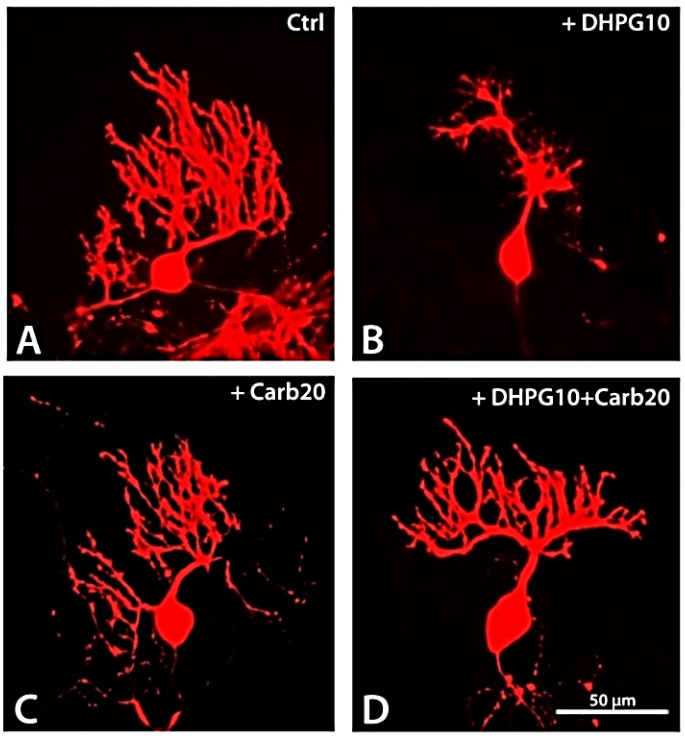

Fig. 7: A) – D) Anti-calbindin stained Purkinje cells from different pharmacological treatment. Purkinje cells from untreated control cultures have a well-defined and profuse dendritic arbor. B) Purkinje cells from slice culture treated with 10 µM DHPG have a dendritic arbor greatly reduced in size with an absence of the distal dendritic branches. C) Purkinje cells from carboxyeosin treated cultures have a dendritic arbor smaller compared to untreated control but larger compared to DHPG treated cultures. D) Co-treatment with 20 µM carboxyeosin provides a partial rescue of the distal dendritic branches of the Purkinje cells. The size of the dendritic arbor is larger compared to DHPG treatment alone. Scale bar = 50 µm.

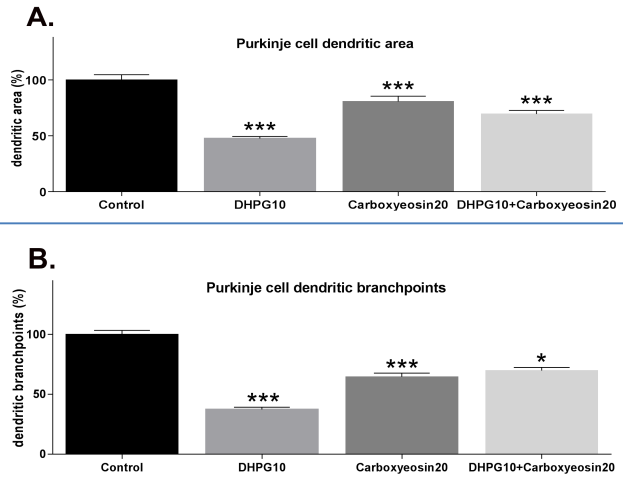

Fig. 8: A)- Size of Purkinje cell dendritic arbors with different pharmacological treatments: The mean size of control Purkinje cells was considered as 100%. Dendritic tree size was reduced to 48% of control with DHPG treatment. Co-treatment with carboxyeosin resulted in a rescue of the dendritic area to 70% of the control value, similar to the size after treatment with carboxyeosin alone. **B)- Number of branch points with the pharmacological treatments:** The mean number of branch points of control Purkinje cells was considered as 100%. The branchpoint number is reduced to 37% in DHPG treated cultures compared to control. Co-treatment with carboxyeosin showed a rescue of the branchpoints to 70% of the control value. These differences were significant with $p < 0.001$ (***). Error bars represent the SEM.

inhibition reduces Purkinje cell dendritic tree size confirms earlier observations with PMCA2 knockout mice. The somewhat unexpected finding that PMCA2 inhibition protects Purkinje cells from mGluR1 mediated dendritic reduction highlights the importance of the calcium equilibrium for the control of Purkinje cell dendritic tree size during dendritic development.

Our previous work has shown that chronic mGlur1 stimulation during Purkinje cell dendritic development in cerebellar slice cultures induces a marked reduction of the Purkinje cell dendritic tree size (Sirzen-Zelenskaya et al., 2006) and Ca^{2+} influx through voltage gated P/Q-type and T-type channel is crucial mediator of the DHPG-induced dendritic tree reduction (Gugger et al., 2012). In agreement with this concept, we had shown that inhibition of P/Q and T-type of calcium channels had a rescuing effect for the Purkinje cell dendrites after DHPG-treatment.

Inhibiting PMCA2 by carboxyeosin will inhibit a major Ca^{2+}-extrusion mechanism and would thus result in increased Ca^{2+} levels. This view is supported by the finding of increased resting levels of intracellular Ca^{2+} in Purkinje cells (Empson et al., 2010) and a reduced size of the Purkinje cell dendritic tree in the PMCA2 knock out mouse (Empson et al., 2007). Our finding of a moderate reduction of Purkinje cell dendritic tree size after carboxyeosin treatment alone is in agreement with the previous observations and confirms the importance of PMCA2 for maintaining the Ca^{2+}-equilibrium in Purkinje cell dendrites. The strong presence of PMCA2 at the plasma membrane of the distal dendritic tree and in dendritic spines of Purkinje cells underlines its important role for calcium homeostasis in the Purkinje cell dendrites. It is very likely that chronically elevated resting calcium levels induced by PMCA2 inhibition through carboxyeosin treatment had an inhibitory effect on dendritic growth. Because the size of the dendritic tree was only moderately reduced, it is likely to assume that the Purkinje cells activated compensatory mechanisms which could to a large degree counteract the reduction of Ca^{2+} extrusion induced by PMCA2 inhibition.

We had previously suggested that an increased Ca^{2+} influx and a rise of the intracellular Ca^{2+}-concentration is the effector pathway for Purkinje cell dendritic reduction after mGluR1 stimulation by DHPG treatment. If this assumption was correct then the protective effect of co-treatment of DHPG with carboxyeosin is somewhat surprising. Normally, a block of a major Ca^{2+} extrusion mechanism would be expected to result in an increased intracellular Ca^{2+} concentration and should lead to a potentiation of the DHPG effect. A possible explanation for our contradictory finding of a protective effect probably comes from the chronic nature of the drug treatments in this study, leading to the activation of compensatory mechanisms within the Purkinje cell dendrites.

In the wriggle mutant mouse, there is a point mutation in the PMCA2 gene resulting in a loss of function phenotype similar to the PMCA2 knockout mouse (Ueno et al., 2002). When

depolarization-induced Ca^{2+} influx into Purkinje cells in these mice was studied, it was found to be greatly reduced quite in contrast to the expectation of an increased Ca^{2+} rise due to the reduced extrusion. The most likely explanation for this finding is a compensatory inactivation and downregulation of voltage-gated Ca^{2+}-channels with a strong reduction of depolarization-induced Ca^{2+} influx as found in these mice (Ueno et al., 2002). In accordance with this explanation, it was shown that there was a dramatic reduction of both the frequency and amplitude of complex spikes and depolarization-induced Ca^{2+} influx in Purkinje cells from PMCA2 knockout mice (Empson et al., 2010). It can be expected that a similar functional inactivation of voltage-gated Ca^{2+} channels did occur with the chronic carboxyeosin treatment. Thus, carboxyeosin treatment can be seen as an effective alternative way to inhibit voltage-gated Ca^{2+} channels and reduce Ca^{2+} influx in Purkinje cells mediated by mGluR1 activation.

4.6. Conclusions

The rescuing effect of carboxyeosin co-treatment for Purkinje cell dendrites with mGluR1 activation found in this study may appear surprising at the first glance, but it confirms our previous findings that a rescue of the Purkinje cell dendritic tree from mGluR1-induced reduction can be achieved by the inhibition of voltage-gated Ca^{2+} channels (Gugger et al., 2012). It further shows that PMCA2 is crucially involved in the maintenance and control of the calcium equilibrium in developing Purkinje cell dendrites and that this equilibrium is critical for the control of dendritic growth and expansion.

Acknowledgements:

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4.7. Supplemental material:

Table-1:

Statistics\conditions	Control	Carboxyeosin10	carboxyeosin20	DHPG10	DHPG10 + Carboxyeosin20
Number of values	58	68	52	91	65
Mean dendritic area	3820	3201	3082	1816	2656
Std. Deviation	1355	1394	1309	658	965
Std. Error of Mean	177	169	181	69	119

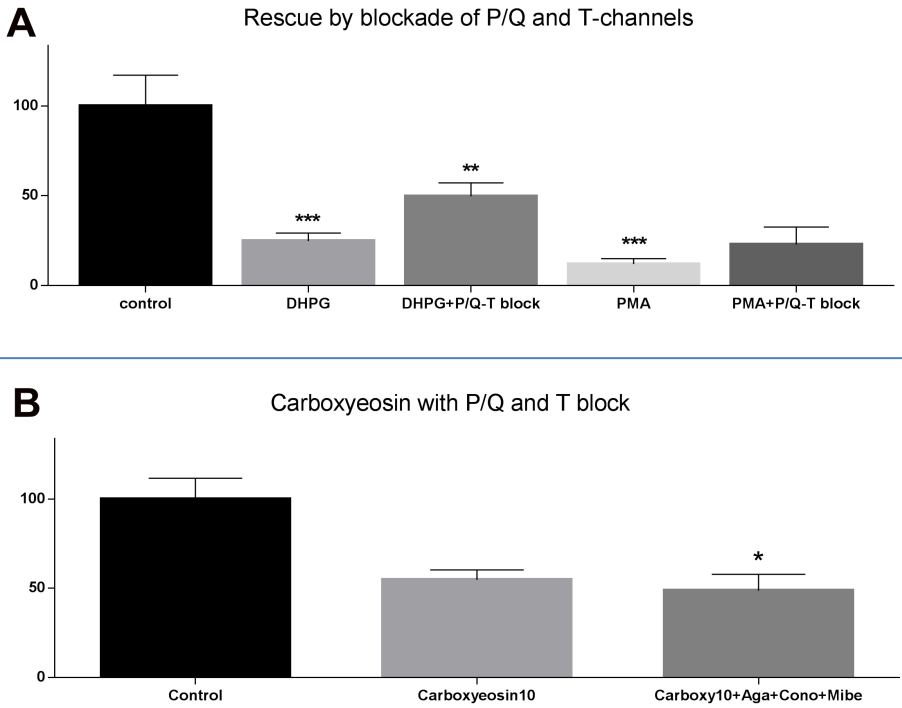
Supplemental Table 1: Measured values for the mean dendritic tree size of Purkinje cells in the different treatment conditions given in square micrometres. The "number of values" indicates the number measured Purkinje cell dendritic trees per treatment condition, the values for standard deviation and standard error of the mean indicate the variance of the data sets.

Table-2:

Statistics\conditions	Control	Carboxyeosin 10	Carboxyeosin 20	DHPG10	DHPG10 + Carboxyeosin 20
Number of values	22	22	22	22	22
Mean branchpoints	30	23	19	11	21
Std. Deviation	5	5	4	3	4
Std. Error of Mean	1	1	1	1	1

Supplemental Table 2: Measured values for the mean dendritic branch points of Purkinje cells from different treatment conditions. The "number of values" indicates the number of quantified Purkinje cells for their dendritic branch points per treatment condition, the values for standard deviation and standard error of the mean indicate the variance of the data sets.

Supplemental Fig. 1:



Supplemental Fig 1: A)- Size of the Purkinje cell dendritic arbours with blockade of P/Q-type and T-type Ca^{2+} channels and mGluR1/PKC stimulation: Blockade of P/Q-type and T-type Ca^{2+} channels with mGluR1 activation does provide a rescue from DHPG mediated dendritic reduction. Similarly, Ca^{2+} channels blockade shows similar dendritic rescue when PKC is activated by PMA. The mean size of control Purkinje cells was considered as 100%. Error bars represent the SEM. One set of experimental data with n= 15. **B)- Size of the Purkinje cell dendritic arbours with blockade of P/Q-type and T-type Ca^{2+} channels and PMCA2 inhibition:** The Purkinje cell dendritic arbours in with blockade of P/Q-type and T-type Ca^{2+} channels in addition to PMCA2 does not have an additive rescuing effect when PMCA2 was inhibited by carboxyeosin. The mean size of control Purkinje cells was considered as 100%. Error bars represent the SEM. One set of experimental data with n= 25.

5. THE NA^+/CA^{2+} EXCHANGER AND PURKINJE CELL DENDRITIC DEVELOPMENT

Chronic pharmacological blockade of the Na^+/Ca^{2+} exchanger modulates the growth and development of the Purkinje cell dendritic arbor in mice cerebellar slice cultures.

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Keywords: Na^+/Ca^{2+} exchanger, mouse, dendritic development, cerebellar organotypic slice cultures

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5.1. Abstract

The Na^+/Ca^{2+} exchanger (NCX) is a bi-directional plasma membrane antiporter involved in Ca^{2+} homeostasis in eukaryotes. NCX has three isoforms, NCX1–3, and all of them are expressed in the cerebellum. Immunostaining on cerebellar slice cultures indicates that NCX is widely expressed in the cerebellum, including expression in Purkinje cells. The pharmacological blockade of the forward mode of NCX (Ca^{2+} efflux mode) by Bepridil moderately inhibited growth and development of Purkinje cell dendritic arbor in cerebellar slice cultures. However, the blockade of the reverse mode (Ca^{2+} influx mode) by KB-R7943 severely reduced the dendritic arbor and induced a morphological change with thickened distal dendrites. The effect of KB-R7943 on dendritic growth was unrelated to the activity of voltage-gated calcium channels and was also apparent in the absence of bioelectrical activity indicating that it was mediated by NCX expressed in Purkinje cells. We have used additional NCX inhibitors including CB-DMB, ORM-10103, SEA0400, YM-244769 and SN-6 which have higher specificity for NCX isoforms and target either the forward, reverse or both modes. These inhibitors caused a strong dendritic reduction similar to that seen with KB-R7943, but did not elicit thickening of distal dendrites. Our findings indicate that disturbance of the NCX-dependent calcium transport in Purkinje cells induces a reduction of dendritic arbor which is presumably caused by changes in the calcium handling, and underline the importance of the calcium equilibrium for the dendritic development in cerebellar Purkinje cells.

5.2. Introduction

Purkinje cells are the principal neurons of the cerebellar cortex and have a unique and intricate dendritic tree. Dynamic changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) are of fundamental importance for neurotransmission, gene expression, cell survival and Purkinje cell dendritic development [Kapfhammer JP, 2004, Fujishima et al., 2012]. Purkinje cells modulate ($[Ca^{2+}]i$), using Ca^{2+}-binding proteins, intracellular Ca^{2+} stores, Ca^{2+}-ATPase, Na^+/Ca^{2+} exchanger (NCX), P/Q-type and T-type Ca^{2+} channels. The extensive dendritic trees of cerebellar Purkinje cells develop postnatally in mice, and several molecules controlling dendritic development have been identified [Tanaka, 2009]. Some of the molecules strongly affecting dendritic development are linked to neuronal activity of Purkinje cells, for example protein kinase Cγ [Metzger and Kapfhammer JP., 2000, Schrenk et al., 2002] or the metabotropic glutamate receptor, mGluR1 [Sirzen-Zelenskaya et al., 2006]. In both cases, the activation causes dendritic reduction which is probably caused by Ca^{2+} overload in dendrites. This phenomenon is likely to be mediated by P/Q and T-type Ca^{2+} channels as

their blockade partially rescues Purkinje cells from dendritic reduction [Gugger et al., 2012]. More recently, we have demonstrated that blockade of the plasma membrane calcium ATPase-2 (PMCA2) moderately inhibits the development of the Purkinje cell dendritic arbor. In contrast, chronic PMCA2 inhibition partially rescues mGluR1-induced dendritic reduction, suggesting that the influx or efflux of calcium plays a pivotal role in the development of the Purkinje cell dendritic arbor [Sherkhane P and Kapfhammer JP., 2013].

The regulation of intracellular Ca^{2+} and Na^{+} ion concentrations is important for maintaining cellular homeostasis. The Na^{+}/Ca^{2+} exchanger (NCX), together with selective ion channels and ATP-dependent pumps controls the physiological cytosolic concentrations of Na^{+} and Ca^{2+} ions [Blaustein MP and Lederer WJ., 1999]. NCX mediates Ca^{2+} and Na^{+} fluxes across the synaptic plasma membrane in bi-directional modes: the forward mode (Ca^{2+} efflux mode) and the reverse mode (Ca^{2+} influx mode) [Blaustein MP and Lederer WJ., 1999, Philipson KD and Nicoll DA., 2000, Tong and Hilgemann, 2004]. The stoichiometry of the exchanger is 3:1 Na^{+}:Ca^{2+} ions respectively [Blaustein MP and Lederer WJ., 1999], although it has been reported that the ion flux ratio can vary from 1:1 to 4:1 depending on the intracellular concentration of Na^{+} and Ca^{2+} ions [Tong and Hilgemann, 2004, Fujioka et al., 2000]. NCX may play an important role in modulating neuronal activity and affecting excitotoxicity [Storozhevykh et al., 1998, Thayer et. al., 2002]. Moreover, NCX-mediated Ca^{2+} influx and removal of intracellular Ca^{2+} can modulate synaptic transmission [Scotti et. al., 1999].

NCX is encoded by the SLC8 gene family and is present in several isoforms [Philipson and Nicoll, 2000, Lytton, 2007, On et al., 2008]. The SLC8 family belongs to the CaCA (Ca^{2+}/cation antiporter) superfamily, which in addition to the NCX family includes four other gene families [Lytton, 2007]. Three different SLC8 genes have been mapped in mammals: SLC8A1 encoding NCX1 [Nicoll et al., 1990], SLC8A2 encoding NCX2 [Lee et al., 1994] and SLC8A3 encoding NCX3 [Kofuji et al., 1994, Quednau et al., 2004]. In addition, SLC8A1 and SLC8A3 undergo alternative splicing [Philipson and Nicoll, 2000, Lytton, 2007, Kofuji et al., 1994, Quednau et al., 2004]. The expression of NCX1-3 and their splice variants is tissue-specific [Nicoll et al., 1996, Quednau et al., 1997]. NCX2 is expressed in the brain and NCX3 is expressed in brain and skeletal muscles, whereas NCX1 is abundantly expressed in heart, brain and kidney [Lytton, 2007].

Purkinje cells have a very high Ca^{2+} binding ratio which endows them with the ability to efficiently handle large $[Ca^{2+}]i$ loads [Fierro and Llano, 1996]. NCX contributes to the $[Ca^{2+}]i$ clearance mechanism along with SERCA pumps and PMCA pumps in Purkinje cells [Fierro et al., 1998].

We have studied the role of NCX transporters for dendritic expansion of Purkinje cells by using isoform-specific and more general pharmacological inhibitors of NCX and tested whether the

blockade of NCX would modulate the growth and development of the Purkinje cell dendritic arbor. Our results indicate that NCX isoforms are expressed in Purkinje cells and that the blockade of NCX activity in organotypic slice cultures results in a marked reduction of Purkinje cell dendritic arbor suggesting that NCX regulation of Purkinje cell calcium equilibrium is an important determinant of dendritic development.

5.3. Materials and Methods

5.3.1. *Organotypic slice cultures*

Animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and were reviewed and permitted by Swiss authorities. Cultures were prepared from B6CF1 mice (CB6) as described previously [Adcock *et al.*, 2004; Kapfhammer, 2005]. Mouse pups of undefined sex were decapitated at postnatal day 8 and their brains were dissected aseptically. The cerebellum was separated in ice-cold preparation medium (minimal essential medium (MEM), 1% glutamax (Gibco, Invitrogen), pH 7.3) and slices of 350 µm thickness were cut with a McIllwain tissue chopper under sterile conditions. Cerebellar slices were separated, transferred to a permeable membrane (Millicell-CM, Millipore) and incubated with incubation medium (50% MEM, 25% Basal Medium Eagle, 25% horse serum, 1% glutamax, 0.65% glucose) with 5% CO_2 at 37°C. The medium was refreshed every 2-3 days.

The following pharmacological compounds were added to the medium at each change for a total of 7 days, starting at 2nd or 3rd day in vitro (DIV): 5 µM Bepridil hydrochloride, 100 µM DL-AP5, 40 µM CNQX, 3 µM YM-244769, 10 µM SN-6 and 2 µM Mibefradil dihydrochlorode (Tocris Bioscience, United Kingdom), 15 µM KB-R7943 and 10 µM Gabazine (Abcam Biochemicals, United Kingdom), CB-DMB and ORM-10103 (Sigma-Aldrich, Germany), 0.1 µM ω-agatoxin IVA, 1 µM ω-conotoxin MVIIC (Smartox Biotechnology, France) and 0.3 µM SEA0400 (Taisho Pharmaceuticals, Japan). Slices were kept in culture for a total of 9-11 days before fixation and immunohistochemistry. Each experiment was performed 3 times, and two mouse pups were used per experiment and 6 mouse pups per experimental condition. There were 18 different conditions in total, and approximately 108 mouse pups were sacrificed during the entire study. The pharmacological treatments did not markedly reduce Purkinje cell survival at the concentrations used in this study (see supplemental Fig. 2).

5.3.2. *Immunohistochemistry*

At DIV 9-11 cultures were fixed in 4% paraformaldehyde for 6-24 hours at 4°C. All reagents were diluted in 100 mM phosphate buffer (PB), pH 7.3. Antibodies were added to the slices in fresh

blocking solution (PB + 3% non-immune goat serum + 0.3% Triton X-100) and incubated overnight at 4°C. After washing in PB, secondary antibodies were added to the slices in PB containing 0.1% Triton X-100 for 2 hours at ambient temperature. For the analysis of Purkinje cell dendritic size, rabbit anti-Calbindin D-28K (Swant, Marly, Switzerland, 1:1000) was used as a primary antibody and goat anti-rabbit Alexa 568 (Molecular Probes, Invitrogen, 1:500) was used as a secondary antibody. For NCX expression studies, rabbit polyclonal anti-NCX1 (Alomone labs, Israel cat #: ANX-011), 1:100 and rabbit polyclonal anti-NCX3 (cat #: TA323836), 1:1000 (Origene, United States) were used as primary antibodies and goat anti-rabbit Alexa 488 (Molecular Probes, Invitrogen, 1:500) was used as secondary antibody. In order to visualize Purkinje cells in NCX expression studies, mouse anti-Calbindin D-28K (Swant, Marly, Switzerland, 1:500) was used as a primary antibody and goat anti-mouse Alexa 568 (Molecular Probes, Invitrogen, 1:500) as secondary antibody.

Stained slices were mounted on superfrost glass slides with coverslip using Vectashield mounting medium for fluorescence. The microscopic observations were made on an Olympus AX-70 microscope equipped with a Spot Insight digital camera. For NCX expression studies, confocal microscopy was performed on an upright laser scanning microscope (Zeiss LSM700) equipped with solid-state lasers. Images were acquired using a Plan Apo N 63× 1.4 NA oil immersion objective (Zeiss) and standard PMT detectors. Optical z-sections were separated by 200 nm. The laser lines 488 and 568 nm were used for excitation. Multichannel imaging was achieved through sequential acquisition of wavelengths frame by frame. Images were further processed for optimization of brightness and contrast with Adobe Photoshop and ImageJ (Fiji) software.

5.3.3. *Quantitative analysis of cultured Purkinje cell dendrites*

The quantification of Purkinje cell dendritic tree size was done as previously described [Sirzen-Zelenskaya et al., 2006, Adcock et al., 2004]. Purkinje cells which had a dendritic arbor not overlapping with neighboring cells were selected for analyses. An image analysis program (Image Pro Express or ImageJ) was used to trace the outline of the Purkinje cell dendritic tree yielding the area covered by the dendritic tree. Purkinje cells were acquired from three independent experiments with an average number of 20 cells per experiment and per growth condition. For detailed information on the number of cells measured for each condition, see supplemental tables 3, 5, 7 and 9.

Purkinje cell dendritic length was measured using ImageJ software by inverting an indexed colour image to binary and then skeletonizing the dendritic arbor, which was quantified as a distance in pixel values. The mean pixel values were converted to micrometers by calibration with a micrometer scale (supplemental tables 11 and 12).

For counting the number of branchpoints, 1-2 sets of experimental data were used with approximately 20-40 cells per condition. The branchpoints were counted manually due to the highly branched and fine morphology of the Purkinje cell dendrites. A single Purkinje cell picture taken with the 20x lens was displayed in high magnification and every branchpoint was counted and marked with a white dot using Adobe Photoshop [Kapfhammer JP and Gugger OS, 2012]. See supplemental tables 4, 6, 8 and 10 for detailed information on the number of images and the analysis.

5.3.4. *Statistical analyses*

The data were analyzed using GraphPad Prism 7.0 software. The mean values of the dendritic tree area, the dendritic length or the branchpoint number of untreated control cultures were set as 100% and the results were expressed as percentage of controls. Error bars represent the standard error of the mean (SEM). The statistical significance of differences in the parameters was assessed by non-parametric analysis of variance (Kruskal-Wallis test) followed by Dunn's post test. For comparisons of single data columns, Mann-Whitney's non-parametric test was used. Confidence intervals were 95%, statistical significance was assumed with $p<0.05$.

5.4. Results

5.4.1. *NCX is expressed in Purkinje cells in cerebellar slice cultures.*

To validate the expression of NCX particularly in Purkinje cell soma and dendrites in cerebellar slice cultures, we performed immunohistochemistry with several anti-NCX antibodies and anti-Calbindin antibody on cerebellar slices after 10 DIV. While some of the commercially available antibodies are supposed to be specific for particular NCX isoforms, we could not unequivocally confirm such a specificity on Western blots from cerebellar slice cultures. Therefore, we consider the used antibodies as staining the NCX isoforms with unknown preferences. We observed that NCX immunoreactivity (IR) was present in the Purkinje cell soma, the stem dendrite and the distal dendrites (Fig. 9B). Purkinje cells were identified by double-staining with anti-calbindin antibody (Fig. 9A and D). Using a different antibody NCX immunoreactivity was mostly present in the cytoplasm of the cell soma (Fig. 9E). Single optical sections from confocal microscopy also confirmed the presence of NCX immunoreactivity in Purkinje cell soma and dendrites (see supplemental Fig. 3).

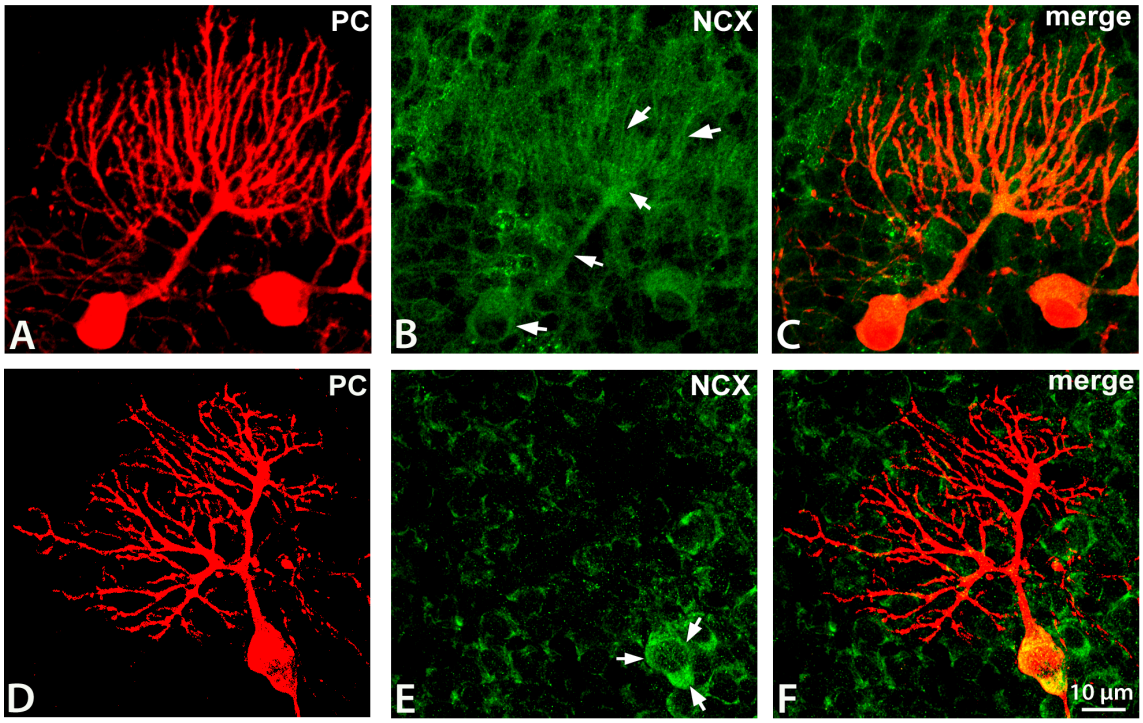

Figure 9: (A-F) Expression of NCX in Purkinje cells: Purkinje cells are shown as a composite of optical sections from the confocal microscope (A, D): Anti-calbindin staining showing the cell soma and the complete dendritic tree. (B) Immunostaining with one polyclonal antibody (Alomone labs, Israel, cat #: ANX-011) NCX was present in the Purkinje cell soma, stem dendrites and distal dendrites of the Purkinje cell (see arrows). (C) Overlay of A) and B). (E) Using another polyclonal antibody (Origene, cat #: TA323836), NCX immunostaining was present prominently in the cytoplasm of the Purkinje cell soma (see arrows) and not detectable in the dendrites. (D) Anti-calbindin staining showing Purkinje cell soma and dendritic tree. ((F) Overlay of D) and E). Scale bar 10 µm.

5.4.2. *Blockade of either the forward mode or the reverse mode of NCX by bepridil and KB-R7943 inhibits the growth and development of the Purkinje cell dendritic arbor.*

The Ca^{2+} exit mode" (forward mode) and "the Ca^{2+} entry mode" (reverse mode) were pharmacologically blocked by bepridil hydrochloride and KB-R7943 respectively. We tested whether the chronic functional inhibition of the forward and reverse mode of NCX with bepridil and KB-R7943 would affect Purkinje cell dendritic development in cerebellar slice cultures.

After 7 days of chronic inhibition of the forward mode by 5 µM bepridil, we could see a moderate reduction of the Purkinje cell dendritic arbor size (Fig. 10B) compared to control cultures (Fig. 10A). The dendritic area was reduced to 83% ±3.06% (Fig. 10E), dendritic length to 79% ±2.06% (Fig. 10F) and the number of branch points to 72% ±2% (Fig. 10G) compared to control. The reverse mode inhibition by 15 µM KB-R7943 more strongly reduced the dendritic arbor size (Fig. 10C). The dendritic area was reduced to 68% ±2.07% (Fig.10E), dendritic length to 57% ±1.28% (Fig. 10F) and the number of branch points to 52% ± 2% (Fig. 10G) compared to control. Furthermore, KB-R7943 treatment resulted in a distinctive morphological change of the distal dendrites which appeared less refined and thickened (compare Figs. 10A and 10C). The combined application of bepridil and KB-

R7943 had an even stronger negative impact on the dendritic development in Purkinje cells. Often only the stem dendrite with few side branches developed (Fig. 10D) and the measurements for the dendritic area, branchpoints and dendritic length were strongly reduced. The dendritic area was reduced to 61% ± 1.87% (Fig. 10E), dendritic length to 50% ±1% (Fig. 10F) and the number of branch points to 49% ±1% (Fig. 10G) compared to control.

5.4.3. *Blockade of P/Q- and T-type of voltage-gated ion channels does not rescue the KB-R7943 - induced dendritic reduction.*

We have shown previously that the dendritic reduction of Purkinje cells seen after mGluR or PKC activation can be partly rescued by blockade of P/Q- and T-type voltage-gated calcium channels [Gugger et al., 2012]. In order to study whether the effects of KB-R7943 may also be mediated by P/Q- and T-type voltage-gated calcium channels, we treated cerebellar slice cultures with KB-R7943 together with P/Q- and T-type calcium channel antagonists for 7 days. To inhibit P/Q-type calcium channels, a combination of 100 nM ω-agatoxin IVA and 1µM ω-conotoxin MVIIC was used as P/Q-block [Mintz et al., 1992a; McDonough et al., 2002, Gugger et al., 2012] and T-type channels were blocked with 2 µM Mibefradil [Huang et al., 2004; McDonough and Bean, 1998].

After 7 days of chronic treatment with P/Q and T-block alone, there was no difference in dendritic morphology and there was no dendritic reduction of the Purkinje cell dendritic arbor in P/Q- and T block treated cultures (Fig. 11B) compared to control (Fig. 11A). KB-R7943 treated slice cultures showed severe reduction of the dendritic arbor size with the formation of thickened distal dendrites (Fig. 11C) as seen earlier (Fig. 10C).

In the quantitative analysis, the dendritic area (94% ±3.29%) and the number of branch points (97% ±3%) were similar in P/Q and T-block cultures compared to control (Fig. 11E, 11F). When KB-R7943 treatment was combined with P/Q and T-block, cell morphology (Fig. 11D) and all measured parameters were similar to those of KB-R7943 treatment alone.

The dendritic area was reduced to 69% ±1.65% with KB-R7943 treatment alone and to 59% ±1.74% in KB-R7943 treatment combined with P/Q and T-block (Fig. 11E), the number of branch points was reduced to 53% ±1% and 54% ±1% respectively (Fig. 11F). These results show that the dendritic reduction and presence of thickened distal dendrites couldn't be rescued by the blockade of P/Q-T type of channels. Our findings confirm that the dendritic morphology and reduction caused by inhibition of reverse mode with KB-R7943 is not related to an activation of P/Q- and T-type voltage gated calcium channels.

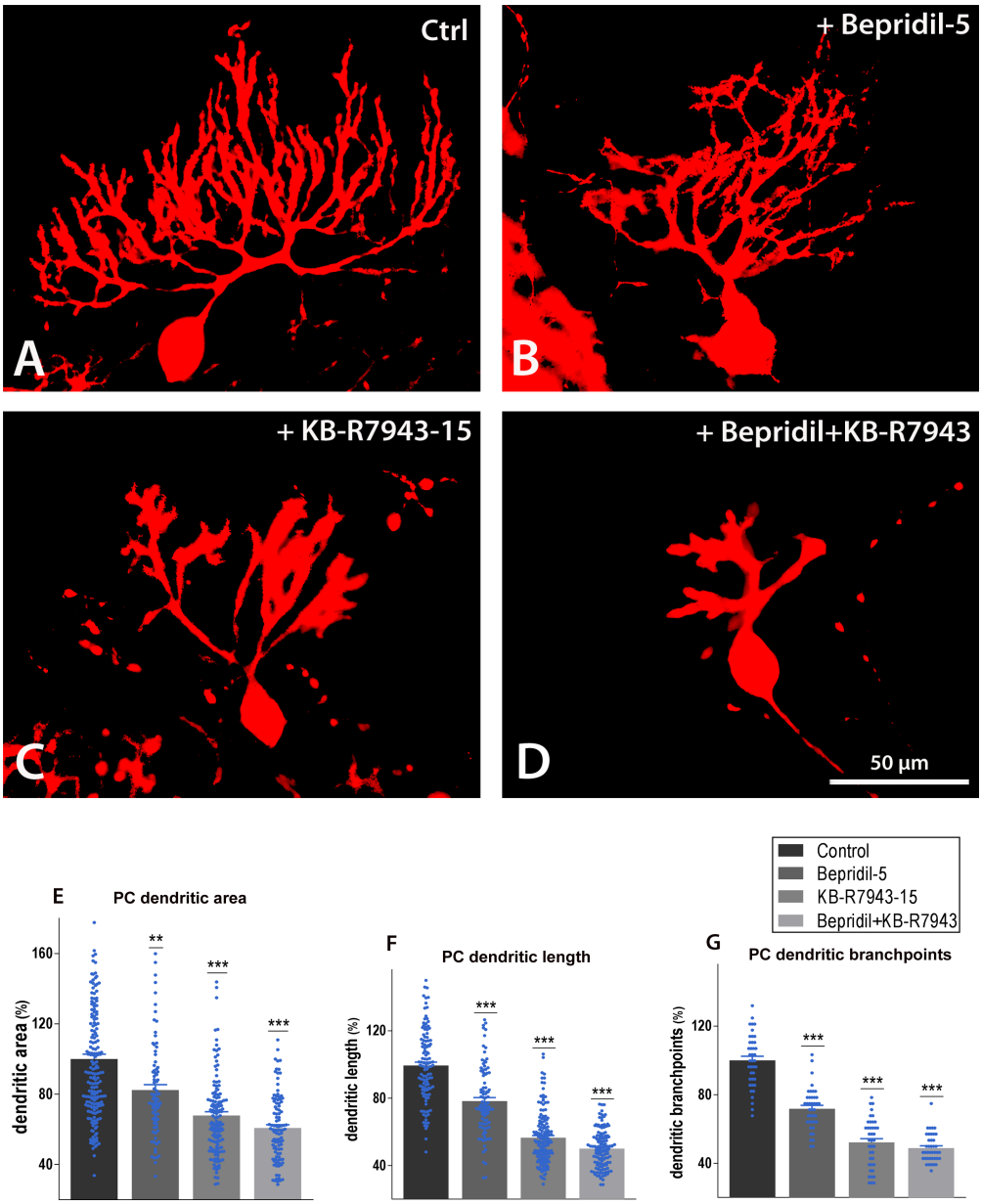

Figure 10: (A-D) Purkinje cell morphology after KB-R7943 and bepridil treatments: (A) Purkinje cell from untreated control cultures with a well-defined and profuse dendritic arbor. (B) Purkinje cell after treatment with 5 µM bepridil with a dendritic arbor slightly reduced in size compared to control. (C) Purkinje cell after treatment with 15 µM KB-R7943. This cell has a severely reduced dendritic arbor and thickened distal dendrites. (D) Purkinje cell after co-treatment with KB-R7943 and bepridil with a severe reduction of the dendritic arbor and thickened distal dendrites. Scale bar 50 µm. **(E) Size of the Purkinje cell dendritic area after different pharmacological treatments:** The mean dendritic area size for control Purkinje cells was set as 100%. Dendritic tree size was reduced to 83% with bepridil hydrochloride treatment compared to control cultures. The dendritic area in KB-R7943 treated cultures was reduced to 68% and co-treatment with bepridil resulted in a reduction to 61% compared to control. **(F) Purkinje cell dendritic length after pharmacological treatments:** The mean value of dendritic length for control Purkinje cells was set as 100%. The dendritic length was reduced to 79% in Bepridil treated cultures and to 57% in KB-R7943 treated cultures compared to control. In bepridil with KB-R7943 co-treatment, dendritic length was reduced to 50% compared to the control value. **(G) Number of branchpoints after pharmacological treatments:** The mean number of branchpoints for control Purkinje cells was set as 100%. The number of branchpoints was reduced to 72% in bepridil treated cultures and to 52% in KB-R7943 treated cultures. After bepridil and KB-R7943 co-treatment the branchpoints were reduced to 49% compared to control. These differences were significant with $P < 0.01$ (**); $P < 0.001$ (***) and error bars represent the SEM.

5.4.4. *KB-R7943-mediated Purkinje cell dendritic reduction is not affected by blockade of AMPA, NMDA or GABA$_A$ receptors.*

Pharmacological treatments in slice cultures affect all cell types present in the culture. The observed effects on the Purkinje cell dendritic arbors could thus be indirect and mediated by changed bioelectrical activity of other cells in the cultures. In order to exclude this possibility, we tested whether the effects seen after the blockade of NCX reverse mode with KB-R7943 is mediated via AMPA, NMDA or GABA$_A$ receptors. The treatment of KB-R7943 (15 µM) was combined with treatments of CNQX (AMPA-receptor antagonist, 40 µM), DL-AP5 (NMDA receptor antagonist, 100 µM) and Gabazine (GABA$_A$ receptor antagonist, 10 µM).

After 7 days of treatment with receptor block of CNQX+DL-AP5+Gabazine, we did not see a difference in the dendritic morphology of the Purkinje cell dendritic arbor compared to control cultures (Figs. 12A, 4B). The dendritic area in "receptor block" treated cultures was equal to control [see also, Adcock *et al.*, 2004; Kapfhammer, 2005]. KB-R7943 treated slice cultures showed a severe reduction of the dendritic arbor size with the formation of thickened distal dendrites (Fig. 12C) as seen before (Fig. 10C and Fig. 11C). KB-R7943 co-treatment with "receptor block" yielded similar results as KB-R7943 treatment alone (Fig. 12D).

Quantitatively, the dendritic area was reduced to 66% ± 2.22% (Fig. 12E) and the number of branchpoints to 57% ± 2% (Fig. 12F) in cultures treated with KB-R7943 alone and to 65% ±2.83% and to 55% ± 1% in cultures with the combined treatment of KB-R7943 and "receptor block" (Fig. 12E, 12F). These results confirm that the dendritic reduction and morphological changes caused by inhibition of the reverse mode of NCX with KB-R7943 are not mediated via AMPA, NMDA or GABA$_A$ receptors, but are most likely due to a direct action of the inhibitor on NCX present in Purkinje cells.

5.4.5. *Inhibition of forward or reverse mode of NCX by several pharmacological compounds mimicks the dendritic reduction, but not the thickening of distal dendrites caused by KB-R7943.*

The inhibition of the reverse mode by KB-R7943 had caused a distinct morphological change with the formation of thickened distal dendrites, in addition to the reduction of the dendritic arbor. In order to clarify whether this phenotype was due to blocking the reverse mode of NCX or whether it could be an effect unrelated to NCX, we used alternative NCX inhibitors targeting different NCX isoforms more specifically and the forward or reverse mode.

The Benzyloxyphenyl derivatives like KB-R7943, SEA0400, SN-6, and YM-244769 have been developed as selective NCX inhibitors [Iwamoto T, 2007]. SEA0400 was reported to be the most potent and selective inhibitor [Matsuda et. al., 2001].

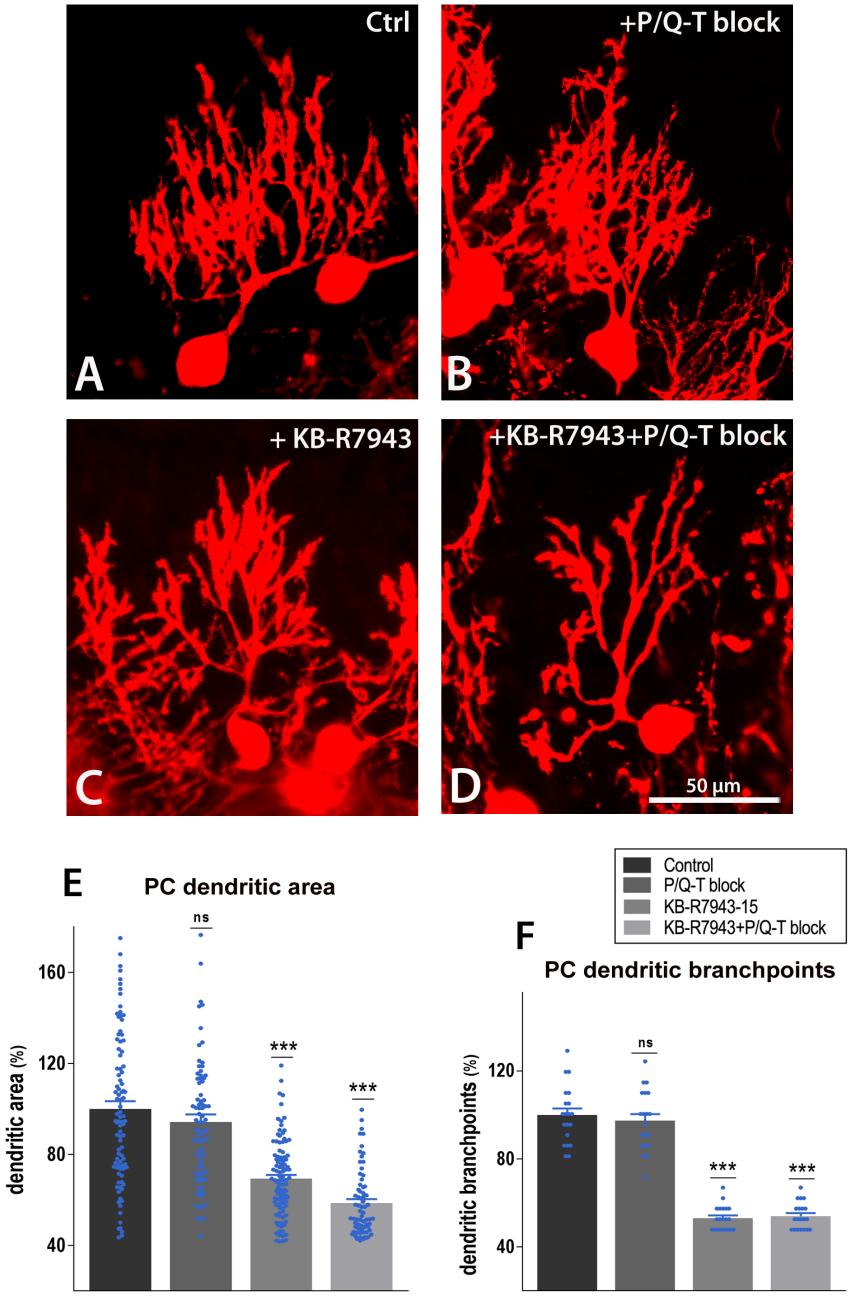

Figure 11: (A-D) Purkinje cell morphology after pharmacological treatments combined with P/Q- and T-block: (A) Purkinje cell from untreated control culture with a well-defined dendritic arbor. (B) Purkinje cell after treatment with a P/Q and T-block with a similar morphology as control Purkinje cells. (C) Purkinje cell after treatment with KB-R7943 with a severely reduced dendritic arbor and thickened distal dendrites. (D) After treatment with KB-R7943 plus P/Q and T-type calcium channel block, Purkinje cells had a similar dendritic morphology seen after KB-R7943 treatment alone. Scale bar 50 µm. **Size of the Purkinje cell dendritic area after pharmacological treatments combined with P/Q- and T-block:** The mean dendritic area size for control Purkinje cells was set as 100%. The dendritic area size was slightly reduced in P/Q- and T-channel block to 94% compared to control. After KB-R7943 treatment dendritic area fell to 69% and to 59% in KB-R7943+P/Q- and T-channel block treated cultures compared to control. **(F) Number of branchpoints after pharmacological treatments combined with P/Q- and T-block:** The mean number of dendritic branchpoints for control Purkinje cells was set as 100%. After P/Q and T-channel block the number was slightly reduced to 97% compared to control. After KB-R7943 treatment branchpoints were reduced to 53% and to 54% in KB-R7943+P/Q- and T-channel block compared to control. These differences were significant with $P < 0.001$ ($***$) and error bars represent the SEM.

These inhibitors have some specificity for individual NCX isoforms, for example KB-R7943 is more specific for NCX3 than NCX1 or NCX2 and selectively inhibits the reverse mode of NCX [Iwamoto T. et al 2001, Iwamoto T and Shigekawa M., 1998], whereas SEA0400 predominantly blocks NCX1, slightly blocks NCX2 and almost doesn't act on NCX3 [Iwamoto T. et al., 2004a]. SN-6 is more specific for NCX1 than NCX2 or NCX3 and has transport mode selectivity for the reverse mode [Iwamoto T. et al., 2004]. YM-244769 is preferentially selective for NCX3 rather than NCX1 or NCX2 and also inhibits the reverse mode [Iwamoto T and Kita S., 2006].

The amiloride derivative CB-DMB inhibits both the forward and the reverse mode of all three NCX isoforms in a concentration-dependent manner with IC_{50} values in the nanomolar range [Secondo A. et.al, 2009]. ORM-10103 is a more recent potent and specific inhibitor of NCX and inhibits both inward and outward currents with IC_{50} values of 780 nM, and 960 nM [Jost N. et al., 2013]. We tested these compounds on cerebellar slice cultures for their effect on the dendritic development of Purkinje cells in parallel with SEA0400, YM-244769 and SN-6 for 7 days in vitro.

The isoform selectivity and the major actions of the pharmacological compounds used in this study are summarized in Table 1. After chronic inhibition of all isoforms and both modes with 5µM CB-DMB, we could see a significant reduction of the Purkinje cell dendritic tree size compared to control (Figs. 13A, 13B). The dendritic area was reduced to 70% ±1.76% (Fig. 13G), and dendritic length to 72% ± 1.76% compared to control (Fig. 5H) and the number of branch points was reduced to 63% ± 2% (Fig. 13I). The inhibition of both modes by 5 µM ORM-10103 also strongly reduced the dendritic arbor size (Fig. 13C). In ORM-10103 treated cultures, the dendritic area was reduced to 68% ±1.61% (Fig. 13G), dendritic length to 72% ±1.71% (Fig. 13B) and the number of branch points to 64% ± 1% (Fig. 13C) compared to control.

The reverse mode inhibition by 0.3 µM SEA0400 also strongly reduced the dendritic arbor size (Fig. 5D). In SEA0400 treated cultures the dendritic area was reduced to 46% ±1.89% (Fig. 13G), dendritic length to 52% ± 1.72% (Fig. 5H) and the number of branch points to 54% ±2% (Fig. 13I) compared to control. Similar results were obtained after inhibition of NCX with YM-244769 (Fig. 5E) and SN-6 (Fig. 13F). In YM-244769 (3 µM) and SN-6 (10 µM) treated cultures dendritic area was reduced to 52% ±1.89% and 54% ± 1.86% (Fig. 13G), dendritic length to 55% ±1.36% and 59% ±1.69% (Fig. 13H) and the number of branch points to 56% ±2% and 53% ±2% (Fig. 13I) respectively.

Taken together, all the different compounds were reducing the size of the dendritic arbor to a similar degree of all measured parameters. In contrast, the conspicuous morphological change of thickened dendrites which was evident in KB-R7943 treated cultures was not observed in YM-244769- or SN-6-treated cultures.

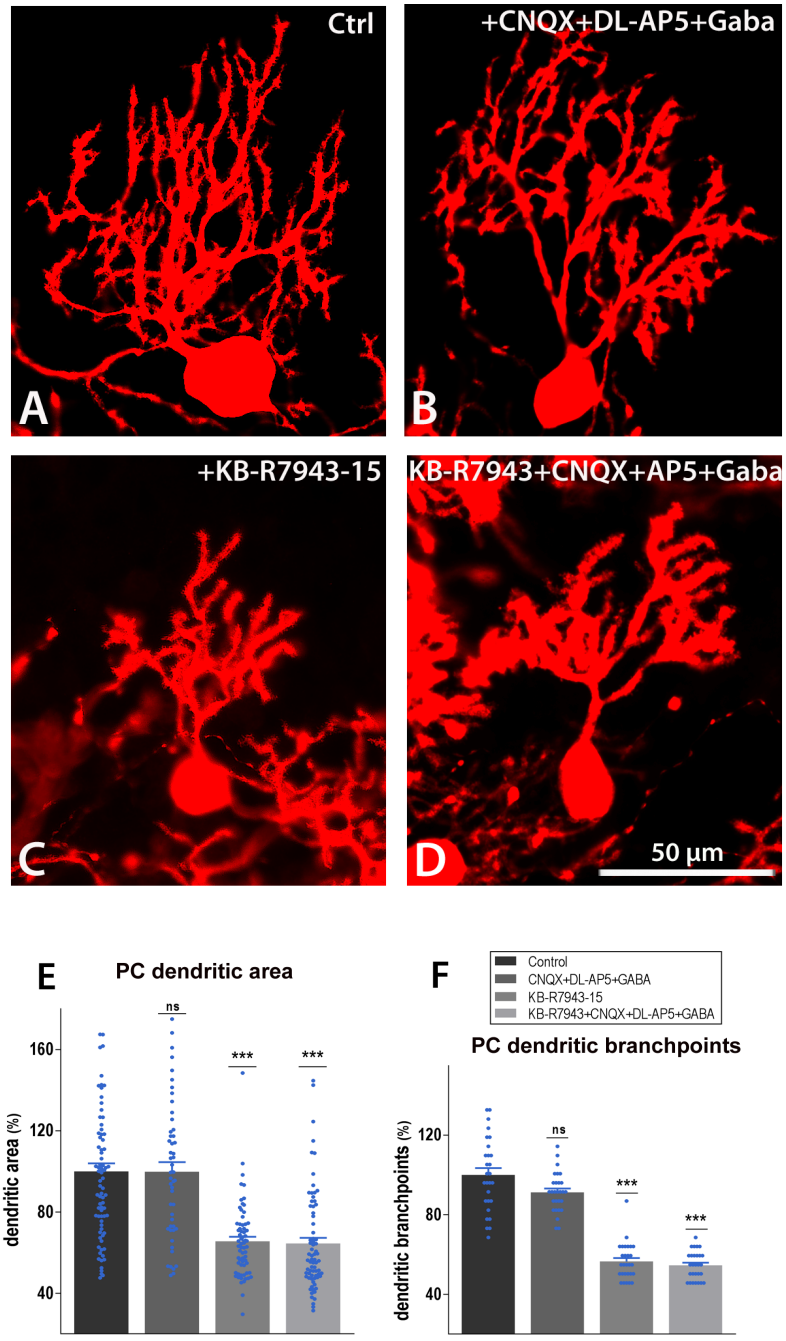

Figure 12: A-D) Purkinje cell morphology after pharmacological treatments together with neurotransmitter receptor block: (A) Purkinje cell from an untreated control culture with a well-defined dendritic arbor. (B) Purkinje cell after treatment with a block of AMPA, NMDA and GABA-A receptor antagonists with a similar morphology as control Purkinje cells. (C) Purkinje cell after treatment with KB-R7943 with a severely reduced dendritic arbor and thickened distal dendrites. (D) After treatment with KB-R7943 plus a block of AMPA, NMDA and GABA-A receptors antagonists, Purkinje cells had a similar dendritic morphology as seen after KB-R7943 treatment alone. Scale bar 50 µm. **(E) Size of the Purkinje cell dendritic area after pharmacological treatments together with neurotransmitter receptor block:** The mean dendritic area size for control Purkinje cells was set as 100%. The dendritic area size was equal to control after CNQX+DL-AP5+Gabazine blockade, 100%. After KB-R7943 treatment, dendritic area fell to 66% in KB-R7943 and to 65% in KB-R7943+CNQX+DL-AP5+Gabazine treated cultures compared to control. **(F) The number of dendritic branchpoints after different pharmacological treatments in NCX blockade:** The mean number of dendritic branchpoints for control Purkinje cells was set as 100%. The number of branchpoints was reduced to 92% in CNQX+DL-AP5+Gabazine blockade. After KB-R7943 treatment branchpoints were reduced to 57% and to 55% in KB-R7943+CNQX+DL-AP5+Gabazine treated cultures. These differences were significant with $P < 0.001$ ($***$) and error bars represent the SEM.

In ORM-10103- and CB-DMB-treated cultures this morphological change was sometimes observed, but much less prominent compared to KB-R7943-treated cultures.

Table 1:

Compound	Forward mode (efflux mode)	Reverse mode (influx mode)	Isoform specificity for Na^+/Ca^{2+} exchanger	References
Bepridil	+	-	unknown	Garcia ML et al., 1988
KB-R7943	-	+	NCX3 > NCX1; NCX2	Iwamoto T et al., 2001
CB-DMB	+	+	NCX1; NCX2; NCX 3	Secondo A et al., 2009
ORM-10103	+	+	NCX1; NCX2; NCX 3	Jost N et al., 2013
SEA0400	-	+	NCX1 > NCX2; NCX3	Iwamoto T et al., 2004a
YM-244769	-	+	NCX3 > NCX1; NCX2	Iwamoto T & Kita S., 2006
SN-6	-	+	NCX1 > NCX2; NCX3	Iwamoto T et al., 2004b

Table 1: This table provides a list of pharmacological inhibitors used in this study. The preference for the transport mode of the Na^+/Ca^{2+} exchanger (either influx or efflux mode) and the isoform specificity is indicated for the different compounds. These data are based on the indicated references.

5.4.6. *Dendritic spines were present on Purkinje cell dendrites after pharmacological treatments in cerebellar slice cultures.*

Purkinje cell dendrites have numerous spines although spine density and shape vary within the dendritic field of the neuron and across cortical lobules [Heinsen and Heinsen, 1983]. In order to see whether the number or shape of the dendritic spines would be strongly affected by pharmacological treatments, we looked at the dendritic spines on high magnification (100x) confocal images. Purkinje cells from cerebellar slice cultures treated with 7 pharmacological inhibitors and from control cultures were photographed. Dendritic spines were present in all experimental conditions and some differences in spine shape and number were apparent (see Fig. 14 A-I). However, we have not further analyzed these subtle differences in spine morphology or number, but show the images in order to confirm that the development of dendritc spines is not compromised by any of the treatments. The thickened distal dendritic phenotype in KB-R7943 treated cultures and after co-treatment with Bepridil is also apparent in these high magnification images (Fig. 14 C; D).

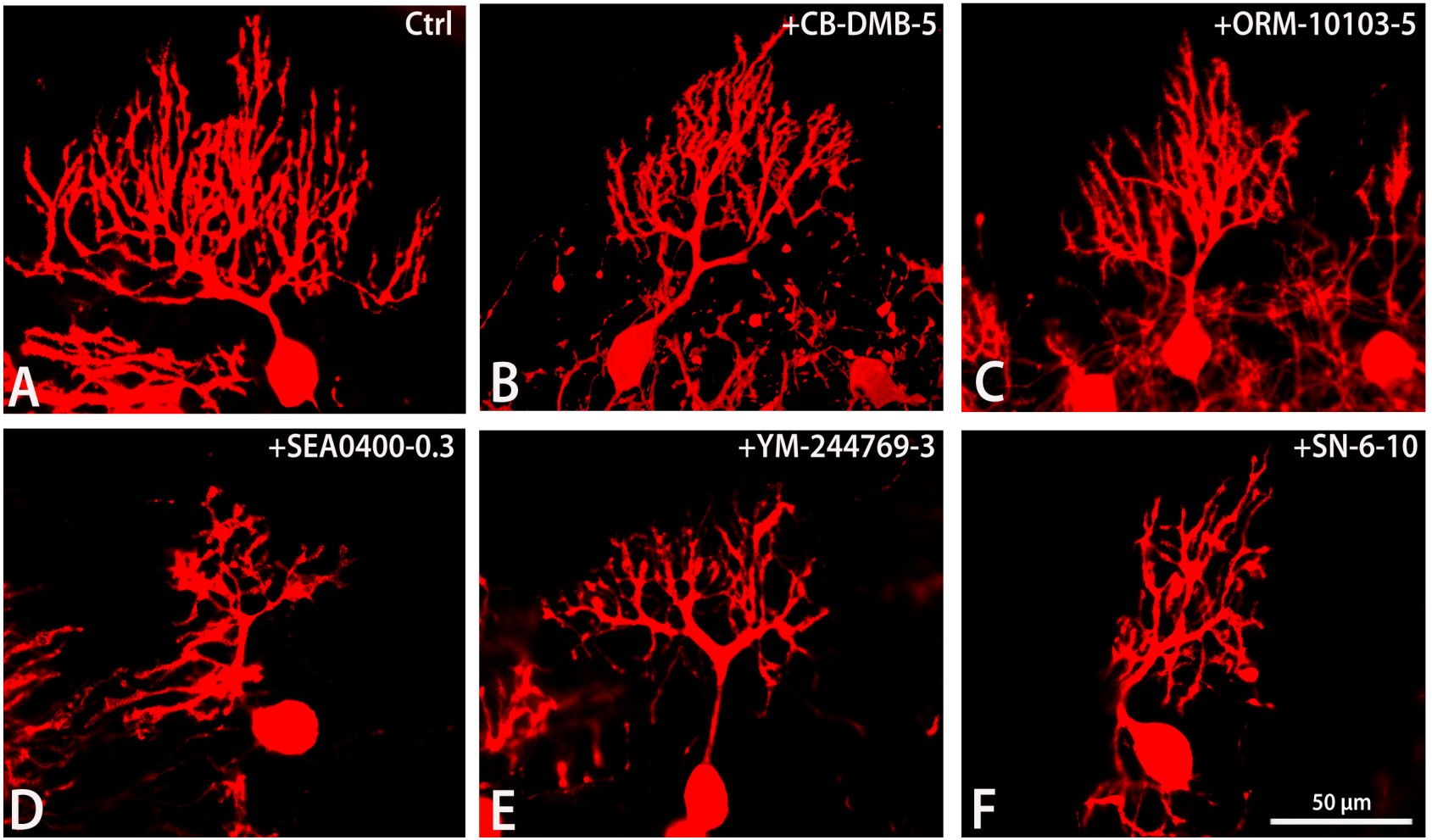

Figure 13: (A-F) Purkinje cell morphology after different pharmacological treatments: (A) Purkinje cell from untreated control cultures with a well-defined dendritic arbor. (B) Purkinje cell after treatment with 5µM CB-DMB with a dendritic arbor reduced in size with less side branches (C) Purkinje cell after treatment with 5µM ORM-10103 with reduced dendritic arbor size and the reduction of side branches. (D) Purkinje cell after treatment with 0.3µM SEA0400 with reduced dendritic arbor size and with few side branches. (E) Purkinje cell after treatment with 3µM YM-244769 with reduced dendritic arbor size and with few side branches. (F) Purkinje cell after treatment with 10µM SN-6 with reduced dendritic arbor size and with few side branches. Scale bar 50 µm.

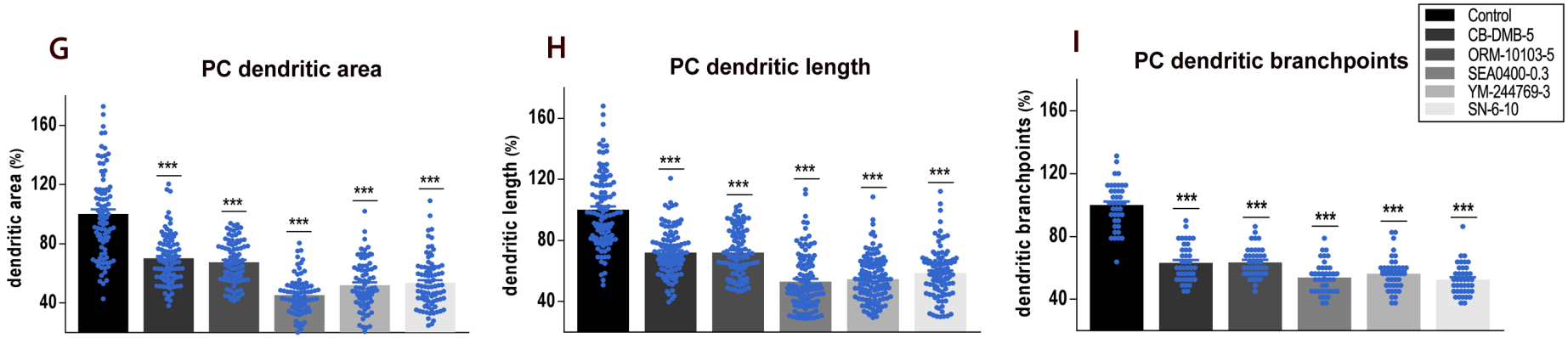

Fig. 13: (G-I) Quantitative data after different pharmacological treatments with NCX inhibitors:
(G) Size of the Purkinje cell dendritic area after different pharmacological treatment: The mean dendritic area size for control Purkinje cells was set as 100%. The dendritic area size was reduced to 70% in CB-DMB, to 68% in ORM-10103, to 46% in SEA0400, to 52% in YM-244769 and to 54% in SN-6 treated cultures compared to control.
(H) Purkinje cell dendritic length after different pharmacological treatment: The mean value of dendritic length for control Purkinje cells was set as 100%. The dendritic length was reduced to 72% in CB-DMB, to 72% in ORM-10103, to 52% in SEA0400, to 55% in YM-244769 and to 59% in SN-6 treated cultures compared to control.
(I) Purkinje cell dendritic branchpoints after different pharmacological treatment: The mean number of branchpoints for control Purkinje cells was set as 100%. The branchpoints number was reduced to 63% in CB-DMB, to 64% in ORM-10103, to 54% in SEA0400, to 56% in YM-244769 and to 53% in SN-6 treated cultures compared to control.
These differences were significant with $P < 0.001$ (***) and error bars represent the SEM.

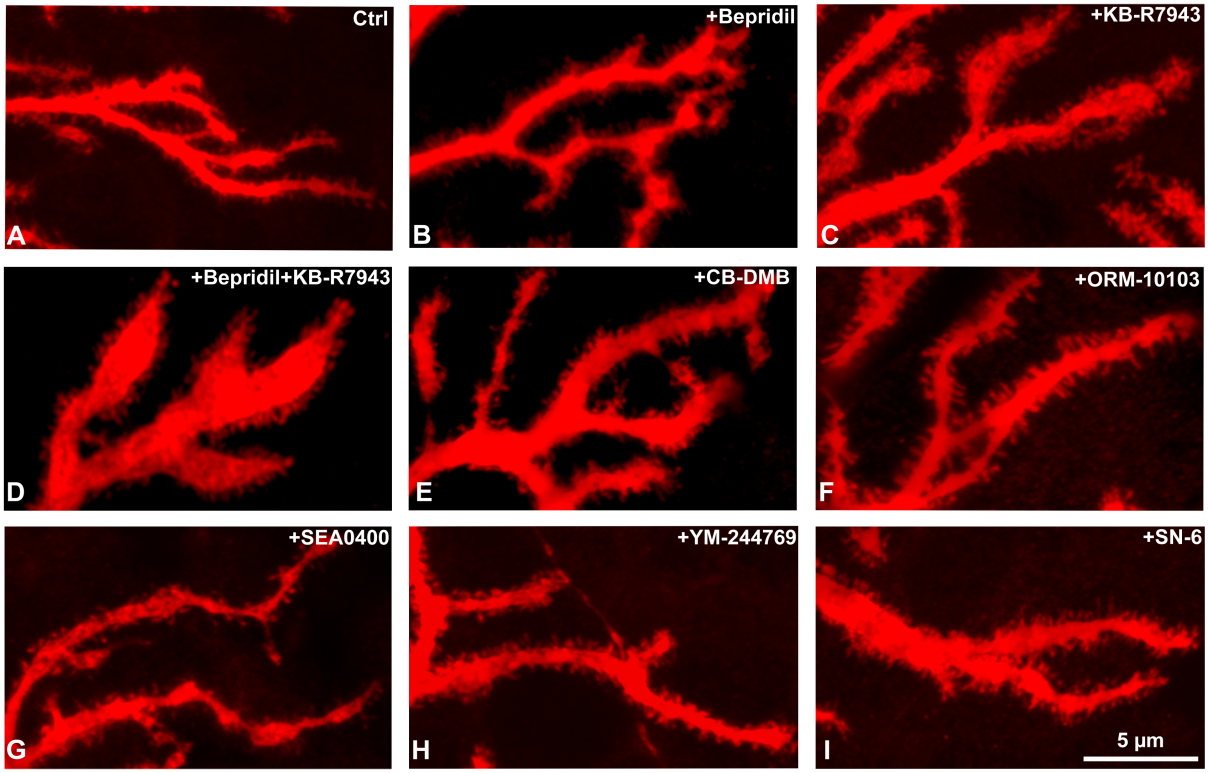

Figure 14: (A-I) Purkinje cell dendritic spines after different pharmacological treatments: (A) Distal dendrite of a Purkinje cell from an untreated control cultures with numerous thin dendritic spines. (B) Purkinje cell distal dendrites with dendritic spines from bepridil treated slices cultures. (C-D) Thickened distal dendrites with dendritic spines from KB-R7943 (C) and KB-R7943 plus bepridil co-treatment (D). (E-F) Distal dendrites from CB-DMB (E) and ORM-10103 (F) treated cultures showing Purkinje cell distal dendrites with dendritic spines appearing slightly thickened compared to control. (G-I) Distal dendrites from SEA0400 (G), YM-244769 (H) and SN-6 (I) with numerous dendritic spines. Scale bar 5 µm.

5.5. Discussion

In this study, we have shown that the sodium-calcium exchanger (NCX) is expressed in Purkinje cells and that its pharmacological inactivation inhibits development of the Purkinje cell dendritic tree in slice cultures. Interestingly, this effect is induced not only by inhibiting predominantly the calcium exit mode (forward mode) of the exchanger, but also by inhibiting the calcium entry/sodium exit mode (reverse mode), or by inhibiting both modes of NCX. Combinations of NCX inhibitors with antagonists of neurotransmitter receptors and voltage-gated calcium channels did not change the effects seen with the blockade of NCX alone indicating that the observed phenotypes are most likely due to the inhibition of NCX in Purkinje cells. The effect was very robust and consistent with a set of different inhibitors targeting preferentially either of the two modes and different molecular isoforms of the exchanger. Our findings indicate that the disturbance of the NCX-dependent calcium transport in Purkinje cells induces changes in the calcium handling of Purkinje cells causing a reduction of the dendritic growth.

5.5.1. *Expression of NCX isoforms in Purkinje cells*

The expression of NCX isoforms in the cerebellum has been studied previously. By in situ hybridization, transcripts of all three NCX isoforms were reported to be expressed in Purkinje cells with NCX1 showing the strongest expression [Papa et al., 2003]. By immunohistochemistry, NCX1 was reported to be strongly expressed in glomeruli of mossy fiber terminals, granule cell dendrites and Golgi cell axons. NCX3 expression was mainly associated with basket cell terminals around Purkinje cell bodies and the axon hillock [Canitano et al., 2002, Papa et al., 2003]. In acutely dissociated cerebellar cells NCX expression was found in Purkinje cells using a non-isoform specific antiserum [Kim et al., 2005]. Our immunostainings on cerebellar slice cultures indicate the widespread expression of NCX in cerebellum and in Purkinje cells. Our results confirm earlier studies and are in agreement with the in situ hybridization data in the Allen Brain Atlas (http://www.brain-map.org/) which show presence of all SLC8A variants in the cerebellum.

5.5.2. *Specificity of the observed effects of pharmacological treatments for NCX*

The widely used inhibitor for the reverse mode, KB-R7943 induced a strong reduction of dendritic tree size in Purkinje cells. Furthermore, it also induced a thickening of the distal dendrites with a strong reduction of small side branches. KB-R7943 has been reported to affect other channels in addition to NCX, in particular to block TRP channels [Kraft, 2007], to activate calcium activated K^+-channels [Liang et al., 2008], to block ryanodine receptors [Barrientos et al., 2009] and to inhibit L-type calcium channels [Cheng et. al., 2011]. In addition, KB-R7943 has been reported to interfere with ion channels, neuronal acetylcholine receptors, the N-methyl-D-aspartate receptor and the norepinephrine transporter [Matsuda T. et al., 2001, Watano T. et al., 1996, Sobolevsky AI. et al., 1999, Pintado AJ. et al., 2000].

In order to exclude that the effects seen with KB-R7943 might be due to non-specific actions rather than to the blockade of NCX, we used a number of additional pharmacological compounds reported to inhibit NCX function; CB-DMB, ORM-10103, SEA0400, YM-244769 and SN-6. Interestingly, all of these compounds irrespective of their reported preference for particular NCX isoforms or transport modes, induced a reduction of the Purkinje cell dendritic tree in the range of approximately 30-50%, i.e. the average size of the dendritic tree was strongly reduced after chronic application of these inhibitors. This inhibition is in the same range as the one seen after KB-R7943 treatment. The finding that five different compounds with diverse chemical structures have a similar effect on dendritic expansion of Purkinje cells makes it unlikely that this was not mediated by inhibition of NCX function. The inhibitor ORM-10103 has recently been shown to be rather specific for NCX with little action on other channels or transporters [Jost et. al., 2013]. Furthermore,

SEA0400 was used in our experiments at a concentration of 0.3 µM strongly suggesting a specific interaction with the NCX transporter. Therefore, the inhibition of Purkinje cell dendritic growth seen with KB-R7943 is likely to be mediated by the inhibition of NCX. In contrast to the reduction of the dendritic tree size, the thickening of individual dendrites as seen with KB-R7943 could not be reproduced with any of the tested compounds. Therefore, it is unlikely that this morphological change is due to the blockade of NCX function. It is much more likely that it results from combining the NCX blockade with other NCX-independent actions of the compound. At the moment, we do not know which of the known unspecific actions of KB-R7943 contribute to this effect, but certainly the morphological phenotype is not due to the blockade of NCX function alone.

5.5.3. *NCX in Purkinje cells is required for changes in dendritic growth*

NCX transporters are widely expressed in many cell types of the cerebellum and important functions of NCX expressed in granule cells have been identified in the parallel fiber-Purkinje cell synapse [Roome et al., 2013]. However, several findings suggest that the observed inhibition of Purkinje cell dendritic expansion is due to the blockade of NCX present in the Purkinje cells and not in other cerebellar cells. First, all three NCX isoforms have been reported to be expressed in Purkinje cells [Canitano et al., 2002, Papa et al., 2003, Kim et al., 2005] and we have confirmed the expression of NCX isoforms in Purkinje cells in our slice culture model system. In order to exclude indirect effects of NCX blockade in other cerebellar cells we have combined treatment of KB-R7943 with blockade of major neurotransmitter receptors inducing a silencing of bioelectrical activity [Baker et al., 1997]. By this co-treatment, it is excluded that Purkinje cells are affected by synaptic activity from neighboring cells in the culture. The reduction of dendritic expansion seen after KB-R7943 treatment was not affected by this co-treatment with the antagonists of major neurotransmitter receptors. While indirect effects through, for example humoral signals of other neurons or the cerebellar glia cannot be completely excluded by these experiments, it is rather unlikely that such signals would remain unchanged by the dramatic change of neuronal activity induced by the blockade of bioelectrical activity. The reduction of dendritic expansion seen after KB-R7943 treatment thus is most likely due to an altered function of the NCX within Purkinje cells.

5.5.4. *Voltage-gated calcium channels are not required for NCX-mediated dendritic reduction*

Previously we have shown that the Purkinje cell dendritic reduction seen after activation of Protein Kinase C or treatment with mGluR agonists, at least in part, is depending on calcium influx through voltage gated calcium channels [Gugger et al., 2012]. In addition, it has been shown that the blockade of another calcium extrusion mechanism; the plasma membrane Ca^{2+}-ATPase2 (PMCA2), results in a compensatory inactivation of voltage gated calcium channels [Sherkhane P and Kapfhammer JP.,

2013-7, Ueno et al., 2002]. We have now combined the blockade of NCX by KB-R7943 with the blockade of P/Q- and T-type channels and have found that the P/Q- and T-block provided no protection from the KB-R7943 induced dendritic reduction. This indicates that calcium influx through voltage-gated calcium channels is not required for the inhibition of dendritic growth by the blockade of NCX. The changes in calcium equilibrium caused by the blockade of NCX do not seem to induce an activation of voltage-gated calcium channels.

5.5.5. *Reduction of Purkinje cell dendritic growth by blockade of NCX*

There is a good evidence that an increased calcium load causes a strong reduction of Purkinje cell dendritic growth and expansion. In the *lurcher (lc)* mouse, dendritic reduction is caused by a mutation in the GluRδ2 channel resulting in a chronic depolarization and increased calcium influx [Zuo et al., 1997, Zanjani et al., 2013], and in the *moonwalker (mwk)* mouse mutant [Becker et al., 2009] the reduction of Purkinje cell dendritic growth is due to increased calcium influx through a mutated TRPC3 channel. The reduction of dendritic expansion after NCX blockade could thus be explained by a chronic elevation of the intracellular calcium concentration due to a reduced efflux after the blockade of NCX function. This explanation would be compatible with the blockade of the forward or calcium exit mode of NCX after which the intracellular calcium concentration is expected to rise. The "forward mode" of NCX is predominantly inhibited by Bepridil, and its treatment resulted in a marked reduction of the dendritic tree size. More unexpectedly, treatment with KB-R7943, an inhibitor of the "reverse mode" was even more effective in reducing the size of the Purkinje cell dendritic tree. Similar results were obtained for treatment with YM-244769 and SN-6, two inhibitors also supposed to block preferentially the reverse mode of NCX [Iwamoto, 2007] and with three recent inhibitors, CB-DMB [Secondo A et al., 2009], SEA0400 [Matsuda et al., 2001] and ORM-10103 [Jost et al., 2013] which are supposed to block both the forward and the reverse mode equally. Taken together all the inhibitors used have shown similar effects on Purkinje cell dendritic expansion, irrespective of target preferentiality to the forward or reverse mode of NCX. One possible explanation for this finding could be, that in the chronic treatment paradigm used in our experiments, all inhibitors will eventually block both directions resulting in a defective calcium handling of the Purkinje cells [Kimura et al., 1999, Tanaka et al., 2002, Niu et al., 2007]. Interestingly, it was previously shown that the blockade of NCX depressed DHPG-induced Ca^{2+} responses in Purkinje cells regardless of NCX modes [Kim et. al., 2007]. The consistent results after using the diverse inhibitors strongly suggests that chronic treatment with any of these inhibitors results in a functional inhibition of NCX currents with the consequence of a marked reduction of the Purkinje cell dendritic arbor. Our results confirm that the maintenance of the calcium equilibrium

and of normal calcium dynamics is of crucial importance for the development of the Purkinje cell dendritic tree, and that the proper function of NCX is required for keeping the calcium homeostasis in Purkinje cells.

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Author Contributions:

PS designed and performed the experiments, interpreted the data and wrote the manuscript. JK designed and supervised the project, assisted in data interpretation and wrote the manuscript.

Conflict of interest:

The authors declare that there is no conflict of interest associated with this study.

Data accessibility:

All data presented in the current manuscript could be obtained from the corresponding author upon request.

5.6. Supplementary data:

Table-3: dendritic area

Statistics\conditions	Control	Bepridil-5	KB-R7943-15	Bepridil-5+KB-R7943-15
Number of values	188	80	137	103
Mean area	3608	2972	2452	2193
Std. Deviation	1361	990	875	688
Std. Error of Mean	99	111	75	68

Table-4: dendritic branchpoints

Statistics\conditions	Control	Bepridil-5	KB-R7943-15	Bepridil-5+KB-R7943-15
Number of values	40	40	40	40
Mean branchpoints	28	20	15	14
Std. Deviation	04	03	04	02
Std. Error of Mean	01	01	01	01

Table-5: dendritic area

Statistics\conditions	Control	CB-DMB-5	ORM-10103-5	SEA0400-0.3	YM-244769-3	SN-6-10
Number of values	90	90	83	78	78	84
Mean area	4152	2908	2801	1881	2159	2223
Std. Deviation	1251	695	612	542	693	708
Std. Error of Mean	132	73	67	62	78	77

Table-6: dendritic branchpoints

Statistics\conditions	Control	CB-DMB-5	ORM-10103-5	SEA0400-0.3	YM-244769-3	SN-6-10
Number of values	40	40	40	40	40	40
Mean branchpoints	27	17	17	14	15	14
Std. Deviation	04	03	03	03	03	03
Std. Error of Mean	01	01	01	01	01	01

Table-7: dendritic area

Statistics\conditions	Control	CNQX+DL-AP5+GABA	KB-R7943-15	KB-R7943+ Receptor block
Number of values	80	50	66	74
Mean area	2913	2909	1911	1879
Std. Deviation	1039	971	527	709
Std. Error of Mean	116	137	65	82

Table-8: dendritic branchpoints

Statistics\conditions	Control	CNQX+DL-AP5+GABA	KB-R7943-15	KB-R7943+ Receptor block
Number of values	28	28	28	28
Mean branchpoints	22	20	12	12
Std. Deviation	04	02	02	02
Std. Error of Mean	01	01	01	01

Table-9: dendritic area

Statistics\conditions	Control	P/Q-T-block	KB-R7943-15	KB-R7943-15+ P/Q-T-block
Number of values	95	82	103	69
Mean area	3259	3074	2260	1910
Std. Deviation	1087	971	546	472
Std. Error of Mean	111	107	54	57

Table-10: dendritic branchpoints

Statistics\conditions	Control	P/Q-T-block	KB-R7943-15	KB-R7943-15+ P/Q-T-block
Number of values	20	20	20	20
Mean branchpoints	21	15	11	11
Std. Deviation	03	02	01	01
Std. Error of Mean	01	01	01	01

Supplemental Table 3, 5, 7 and 9:
Measured values for the mean dendritic area of Purkinje cells from different treatment conditions. The "number of Purkinje cells " indicates the number of quantified Purkinje cells for their dendritic area per treatment condition in square micrometer (μm^2), the values for standard deviation and standard error of the mean indicate the variance of the data sets.

Supplemental Table 4, 6, 8 and 10:
Measured values for the mean dendritic branch points of Purkinje cells from different treatment conditions. The "number of Purkinje cells" indicates the number of quantified Purkinje cells for their dendritic branch points per treatment condition, the values for standard deviation and standard error of the mean indicate the variance of the data sets.

Table-11: dendritic Length in µm (at 20x lens)

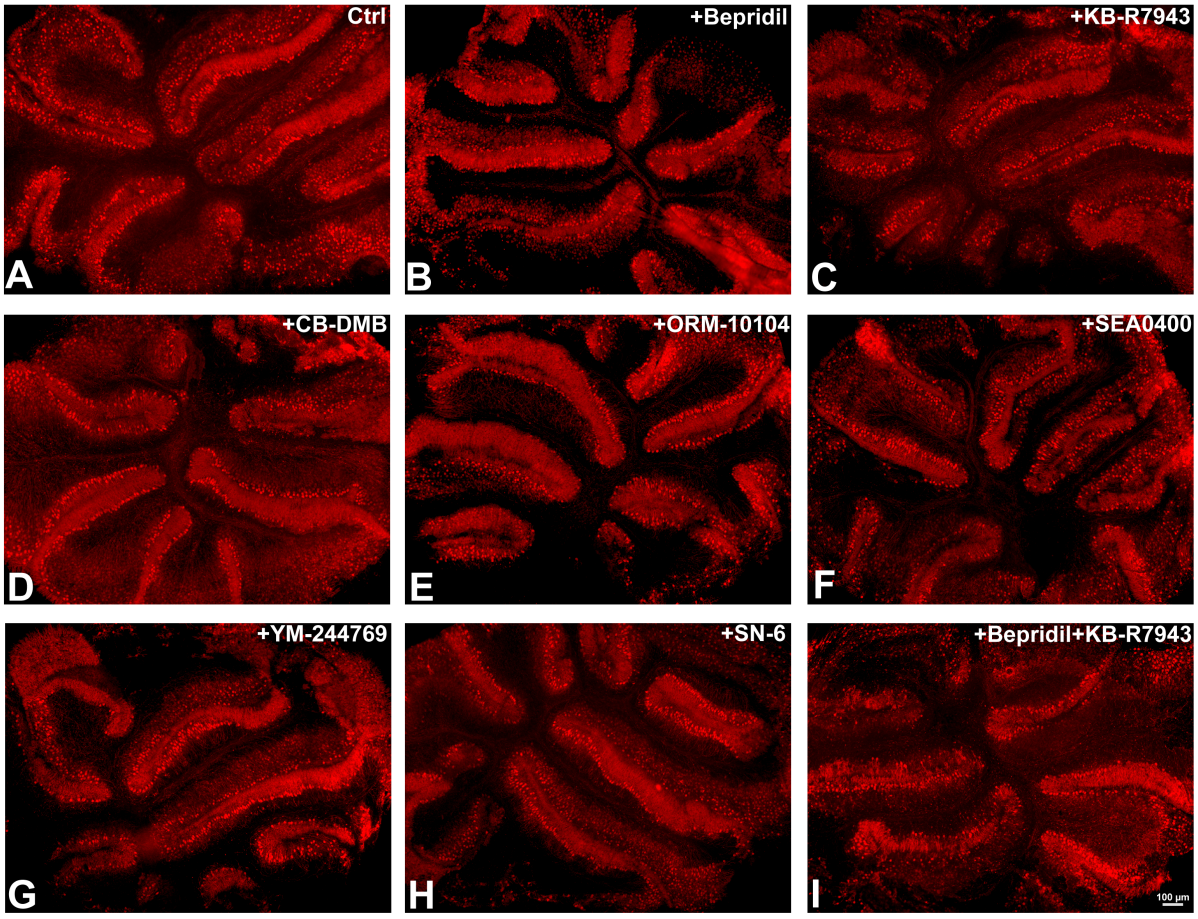
Statistics\conditions	Control	Bepridil-5	KB-R7943-15	Bepridil-5+KB-R7943-15
Number of values	116	95	143	125
Mean dendritic length	1286	1007	727	645
Std. Deviation	289	260	198	144
Std. Error of Mean	27	27	17	13

Table-12: dendritic Length in µm (at 20x lens)

Statistics\conditions	Control	CB-DMB-5	ORM-10103-5	SEA0400-0.3	YM-244769-3	SN-6-10
Number of values	110	103	96	112	117	96
Mean dendritic length	1371	988	988	728	750	803
Std. Deviation	328	209	213	250	203	227
Std. Error of Mean	31	21	22	24	19	23

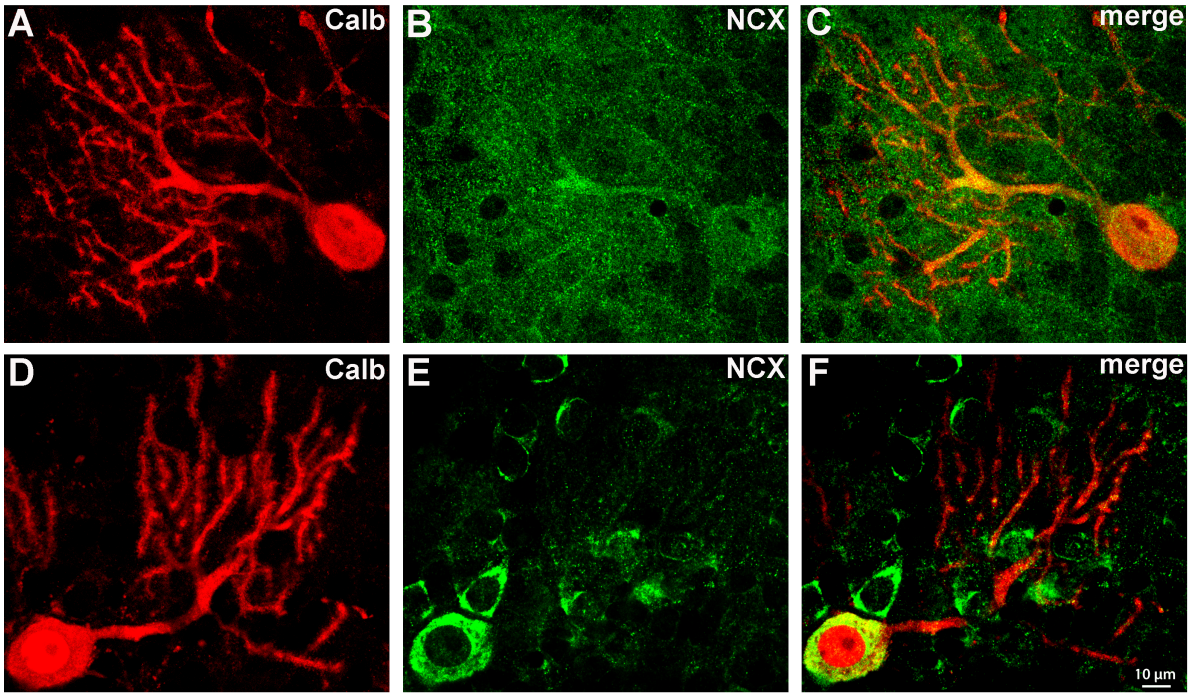
Supplemental Table 11 and 12: Measured values for the mean dendritic length in micrometre (in µm) of Purkinje cells from different treatment conditions. The "number of Purkinje cells" indicates the number of quantified Purkinje cells for their dendritic length per treatment condition in micrometre (µm), the values for standard deviation and standard error of the mean indicate the variance of the data sets.

Supplemental Fig. 2



Suppl. Fig. 2: Purkinje cell survival after different pharmacological treatments: Mouse cerebellar slices after immunohistochemistry with anti-Calbindin primary antibody and Alexa 568 secondary antibody. The images were taken at 4x objective with higher exposer time. In most experimental conditions, Purkinje cells survival was not compromised by the pharmacological treatments. However, in the co-treatment of bepridil and KB-R7943, Purkinje cell survival was slightly affected. Scale bar 100 µm.

Supplemental Fig. 3



Suppl. Fig. 3: NCX expression in Purkinje cells: Confocal images from cerebellar slice cultures after immunostaining with anti-calbindin and anti-NCX antibodies. Purkinje cells were identified by labelling with anti-calbindin antibody (A; D). An antibody from Alomone labs (ANX-011) shows expression in the cell soma, stem and distal dendrites (A; C), anti-NCX antibody from Origene (TA323836) shows expression mostly in the cell soma (E; F) of Purkinje cells. These images are single optical section from confocal microscopy and confirm the presence of NCX immunoreactivity in Purkinje cells. Scale bar 10 µm.

6. UNPUBLISHED DATA

6.1. Expression of NCX isoforms in molecular interneurons

In immunostaining of NCX isoforms in Purkinje cells, many cerebellar cell types appeared to be expressing NCX isoforms. In order to verify whether molecular interneurons or cerebellar granules cells also express NCX isoforms, we used NeuN; neuronal marker for granule cell nuclei and Parvalbumin antibodies to stain granule cells and basket cells respectively. Consistent with the expression of NCX isoforms in Purkinje cells in earlier Fig.9, we could see expression of NCX isoforms in basket cells and presumably in Lugaro or Golgi cells when stained with Parvalbumin antibody (Fig. 15). In NeuN staining, NCX isoforms are expressed in cytoplasm of granule cells (Fig. 16).

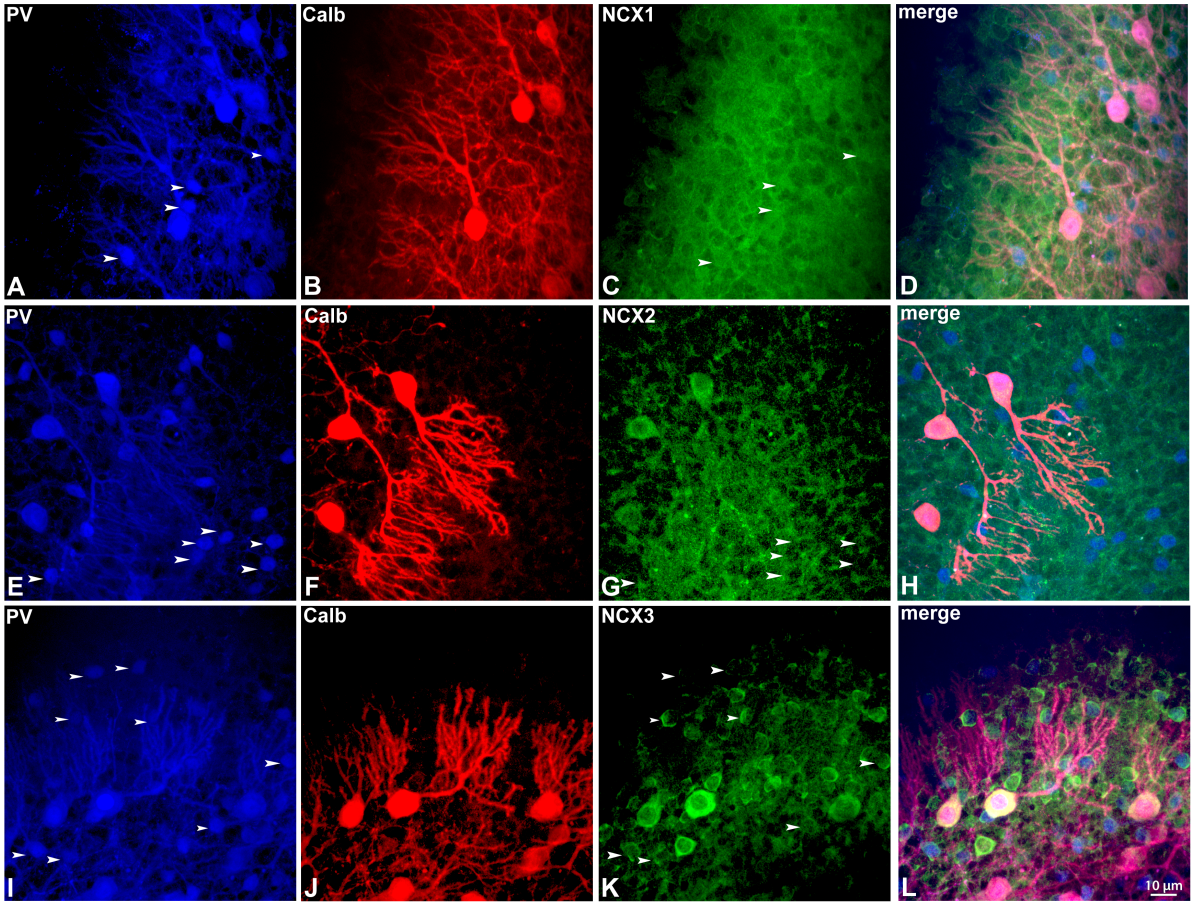


Fig. 15: Expression of Parvalbumin and NCX isoforms in cerebellar interneurons: Confocal images were captured from slices of a P8 mouse cerebellum after triple staining with Parvalbumin, Calbindin and NCX antibodies. PV-expressing cells showed as blue A, E and I in left hand panel. Cells expressing PV and CB appear pink in merge panel at right hand panel are identified as Purkinje cells (B, F and J). Blue stained cells seen in the vicinity of Purkinje cell are identified as interneurons, which express Parvalbumin, but not Calbindin. At P8, PV-containing interneurons are located in close proximity to the Purkinje cell layer and could be identified as basket cells. Images G and K in green represent the expression of NCX1-3 in cerebellum which appears to be in cell soma of Purkinje cells (except NCX1 which is also expressed in stem and distal dendrites) and basket cells. NCX3 appears to be expressed strongly in other cell types presumably Stellate cells and Golgi cells in image K. Scale bar 10 µm.

Figure. 16

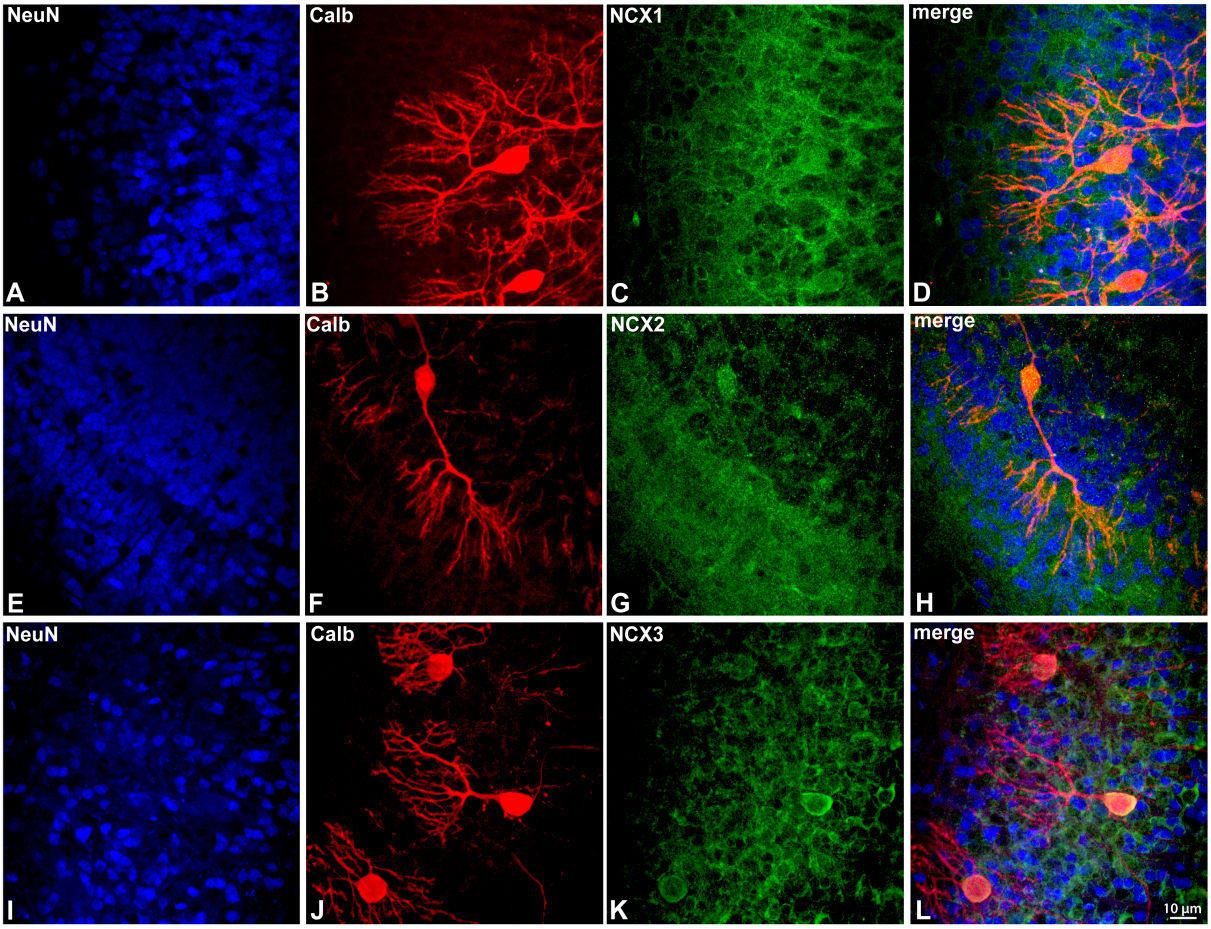


Fig. 16: Expression of NCX isoforms in cerebellar granule cells and Purkinje cells: Confocal images were captured from slices of a P8 mouse cerebellum after triple staining with NeuN; neuronal marker for granule cell nuclei, Calbindin and NCX antibodies. NeuN stains nuclei of granule cells shown in blue A, E and I in left hand panel. Cells expressing NCX and Calbindin appear yellow in the merge panel at the right (D,H and L). Calbindin stained Purkinje cell in red (B, F and J) and C, G and K in green represent the expression of NCX1-3 in cerebellum particularly in cell soma of Purkinje cells (except NCX1 which is also expressed in stem and distal dendrites) and in cytoplasm of granule cells. Scale bar 10 µm.

7. GENERAL DISCUSSION

7.1. *What's the importance of PMCA2 and Na^+/Ca^{2+} exchanger in Purkinje cell dendritic development?*

The plasma membrane (PM) Ca^{2+} ATPase and the Na^+ / Ca^{2+}exchanger are two major antiporters involved in Ca^{2+} extrusion from the cell to the extracellular environment (Carafoli, 1987; Khananshvili, 1998; Brini and Carafoli, 2011). Even though both antiporters are co-expressed in many cell types, their contribution to calcium homeostasis depends on the functional state of the cell (Carafoli, 1987; Berridge et al., 2003; Brini and Carafoli, 2011). The plasma membrane Ca^{2+}-ATPase is a "house-keeping" mechanism that creates and maintains a primary Ca^{2+} gradient across the cell membrane, whereas the Na^+/Ca^{2+} exchanger responds to transient changes in $[Ca^{2+}]i$ under various regulatory conditions (Carafoli, 1987; Khananshvili, 1998; Brini and Carafoli, 2011). The plasma membrane Ca^{2+}-ATPases (PMCAs) are high-affinity calcium pumps that contribute to the maintenance of intracellular Ca^{2+} homeostasis by exporting Ca^{2+} from the cytosol to the extracellular environment. Their distribution is tissue- and cell-specific and undergoes regulation during cell development and differentiation (Marisa Brini, 2009).

Out of the four genes, only one encoding PMCA2 has been linked to diseases in mammals, with several spontaneous mutations that cause deafness and ataxia. A few other human disease phenotypes like hearing loss, cardiac function and infertility are also reported to be associated with PMCA function.

In cerebellar slice cultures, we confirmed the PMCA2 expression in Purkinje cells, particularly in distal dendrites and dendritic spines (Sherkhane and Kapfhammer, 2013). This finding was in line with the earlier observations of PMCA2 expression in Purkinje cells (Filoteo et al., 1997; Hilman et al., 1996; Burette et al., 2009). PMCA2 inhibition by carboxyeosin negatively affected Purkinje cell dendritic arbor development (see Fig. 5E) confirming earlier observations in PMCA2 knockout mice in which the Purkinje cells have a reduced size with a stunted dendritic tree (Empson et al., 2010).

The co-application of DHPG with carboxyeosin in cerebellar slice cultures had a strong rescuing effect for the Purkinje cell dendritic tree after mGluR1 stimulation by DHPG treatment (Fig. 7D). We assume that co-treatment of PMCA2 inhibitor with DHPG leads to a compensatory inactivation of P/Q-type and T-type channels that rescues the dendritic arbor from DHPG-induced reduction (Sherkhane and Kapfhammer, 2013). This view is based on the findings by Uno et al., 2002.

As shown in supplemental Fig. 1A, DHPG induced dendritic reduction was rescued by the blockade of voltage gated P/Q-type and T-type channels confirming the observations by Gugger et al., 2012. In addition, blockade of the voltage gated Ca^{2+} channels combined with PMCA2 inhibition was not having any rescuing effect on the dendritic reduction caused by carboxyeosin (Suppl. Fig. 1B). These findings highlight the importance of PMCA2 function in calcium homeostasis within the Purkinje cells and its role in the dendritic development of cerebellar Purkinje cells.

The Na^+/Ca^{2+} exchanger (NCX) is a Ca^{2+} extrusion antiporter that is powered by the energy of the Na^+ gradient across the cell membrane, which is ultimately derived from the Na^+/K^+ ATPase activity. Since NCX mediates an electrogenic stoichiometry of ion-exchange ($3Na^+$: $1Ca^{2+}$), it can operate either in forward (Ca^{2+}-efflux) or reverse (Ca^{2+}-influx) mode, depending on intracellular and extracellular Na^+/Ca^{2+} ion concentration and the membrane potential (Reeves and Hale, 1984; Blaustein and Lederer, 1999). Three NCX isoforms have been identified in mammals sharing 70% sequence homology and they are expressed in various tissues.

Immunostainings performed on cerebellar slice cultures with anti-NCX antibodies confirmed a widespread expression of NCX in cerebellum and in Purkinje cells. The anti-NCX1 antibody showed strong NCX immunoreactivity in the cytoplasm of the cell soma, stem dendrites and distal dendrites (see Fig. 9B). On the other hand, anti-NCX3 antibody showed strong expression limited to the cytoplasm of the cell soma of Purkinje cells (see Fig. 9E). A widespread expression of NCX was also evident in cerebellar granule cells and molecular interneurons, particularly in basket cells and stellate cells (Fig. 15 & 16 C; G & K). This widespread and general expression pattern is quite distinct from that of PMCA2 which is concentrated at the distal dendrites and the Purkinje cell - parallel fiber synapses suggesting a more general function of NCX not directly related to synaptic transmission.

Bepridil causes a partial inhibition of Na^+-dependent Ca^{2+} uptake and a complete block of Na^+-dependent Ca^{2+} efflux (Garcia et al., 1988). Blockade of the forward mode of Na^+/Ca^{2+} exchanger by bepridil in cerebellar slice cultures caused a moderate reduction of the Purkinje cell dendritic arbor (Fig. 10B). On the other hand, the widely used inhibitor for the reverse mode of NCX, KB-R7943 induced a more pronounced reduction of the dendritic arbor of Purkinje cells with a morphological change of thickened distal dendrites (Fig. 10C). We assume that the thickened distal dendritic phenotype is due to unspecific actions of KB-R7943 and is unrelated to NCX transport function. The morphological change of thickened distal dendrites seen after KB-R7943 treatment was not reproducible when cerebellar slices were treated with new and more specific pharmacological inhibitors. The new compounds like CB-DMB, ORM-10103, SEA0400, YM-244769 and SN-6 are diverse in their isoform specificity, mode of action on reverse mode or on both the modes. NCX

inhibition by these compounds, irrespective of their reported preference for particular NCX isoforms or transport modes, induced a reduction of the Purkinje cell dendritic arbor without producing thickened distal dendrites (Fig. 13).

The activation of Protein Kinase C or mGluR1 by the agonists PMA and DHPG leads to Purkinje cell dendritic reduction and this is partly due to the influx of calcium through voltage gated calcium channels because the dendritic reduction caused by PKC or mGluR1 is partially rescued after the blockade of P/Q- and T-type channels (Gugger et al., 2012). In contrast, that dendritic reduction caused by blockade of NCX was not mediated by P/Q- and T-type channels. Co-treatment of KB-R7943 with P/Q- and T-block did not rescue the dendritic reduction caused by KB-R7943, indicating that voltage-gated calcium channels weren't activated by the NCX blockade and dendritic reduction caused by this blockade was independent of P/Q- and T-type calcium channels (Fig. 11).

Further, we tested whether the observed dendritic reduction was due the blockade of endogenous NCX in Purkinje cells or NCX expressed in other cerebellar cells. The blockade of major neurotransmitter receptors (antagonist of AMPA, NMDA and $GABA_A$) combined with KB-R7943 did not modify the reduction of the dendritic arbor induced by NCX blockade (Fig. 12), confirming that dendritic reduction seen after KB-R7943 was most likely due to an altered function of NCX within the Purkinje cells.

We also looked at the dendritic spines present on Purkinje cell dendrites after treatment with different pharmacological inhibitors of NCX. The dendritic spines were present in all experimental conditions after the treatment (Fig. 14). Some changes in the size, shapes and number of dendritic spines were apparent, but they weren't further analyzed due to the scope of the study.

To conclude, the Na^+/Ca^{2+} exchanger is an important Ca^{2+} antiporter that regulates calcium equilibrium in cerebellar Purkinje cells. A disruption to its function impairs normal calcium handling within the Purkinje cells causing dendritic reduction of Purkinje cells in cerebellar slice cultures.

While the subcellular expression and the function of PMCA2 and NCX in Purkinje cells are quite distinct, the blockade of their function yielded rather similar results. In both cases, there was a moderate reduction of the size of the dendritic tree and the number of branchpoints ranging from approx. 25% (blockade of PMCA2, blockade of forward mode of NCX) to 50% (blockade of reverse mode or both modes of NCX). The protective effect of blockade of PMCA2 for mGluR1 induced dendritic reduction was not present with blockade of NCX. A possible explanation for the similar effects might be that the regulation of the calcium equilibrium in Purkinje cells is complex and involves diverse mechanisms. Blocking one mechanism still allows the Purkinje cells to evoke compensatory mechanisms, as for example the inactivation of the P/Q and T-type channels in case of

PMCA2 blockade. This is not surprising given the chronic nature of our pharmacological treatments. Using such compensatory mechanisms, Purkinje cells do survive and even develop dendritic trees of normal shape. Nevertheless, the loss of one or more of the regulatory mechanisms will lead to a less precise calcium regulation and probably prolonged periods with some dysregulation with the consequence of a reduced size and complexity of the dendritic tree as observed in our experiments. The similarity of the observed outcome of the different treatments could therefore be explained as "Purkinje cell in compensation mode" which would be similar for all the treatments used in this study.

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8. MATERIALS AND METHODS

8.1 Cerebellar slice cultures

8.1.1. Media and prearrangements for cerebellar slice cultures

All ingredients were sterile and media were prepared in a laminar flow cabinet in sterile condition.

Ingredients for 200 ml preparation medium (PM):

- 100 ml minimal essential medium (MEM) (Gibco 11012), in two-fold concentration
- 98 ml Aqua bidest
- 1 ml Glutamax (Gibco 35050)
- 1N NaOH or 1N HCl to adjust pH to 7.2-7.4

Ingredients for 100 ml incubation medium:

- 25 ml MEM (Gibco 11012), in twofold concentration
- 23,5 ml Aqua bidest
- 25 ml Basal Medium Eagle, with Earl's salt, without glutamine (Gibco 21400)
- 25 ml horse serum, heat-inactivated (Gibco 26050)
- 1 ml Glutamax (Gibco 35050)
- 700 µl of a 10% glucose solution
- 1N NaOH or 1N HCl to adjust pH to 7.2-7.4

Pre-arrangements:

- A sterilized razor blade (with 100% ethanol) was installed on a McIllwain tissue chopper.
- Petri dishes (Greiner bio-one 627102, 35mm) were filled with 5 ml ice-cold preparation medium and stored at 4 °C until the dissections. 2-3 dishes per mouse were required.
- 750µl of incubation medium was pipetted in each well of a tissue culture plate (6 wells, Falcon 353046). The plate was placed in an incubator providing a humidified air with 5% CO_2 at 37 °C. One plate holds cultures from 3 mice.
- Surgical instruments were sterilized and placed ready.

8.1.2. ***Procedure***

Animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and were reviewed and permitted by Swiss authorities. Cultures were prepared from B6CF1 mice (CB6) as described previously (Adcock *et al.*, 2004; Kapfhammer, 2005; 2010; Kapfhammer and Gugger., 2012) and incubated according to the static method (Stoppini et al., 1991).

All steps were carried out in the aseptic environment of a horizontal laminar flow cabinet (BDK, Germany).

- A P8 mouse pup was decapitated and the head was sprayed with 70% ethanol.
- The skull was carefully opened in the sterile workbench.
- The cerebellum was removed together with surrounding brain structures (*colliculi inferiores, pons, medulla oblongata*) and placed immediately in a dish filled with ice-cold preparation medium. All further steps were carried out under a stereomicroscope (Zeiss, stemi2000).
- The cerebellum was isolated by cutting the cerebellar peduncles and removing most of the meninges from the surface.
- The cerebellum was placed on the tissue chopper and sagittal slices (350 µm) were cut.
- The sliced cerebellum was placed in a fresh dish with cold preparation medium and the slices were separated from each other. One cerebellum usually yielded 15-18 slices.
- The bottom membranes of two tissue culture inserts (Millicell CM, Millipore PICM 03050) were moistened with preparation medium.
- The slices were carefully laid on the membrane. Both culture inserts should contain about the same amount of slices from different regions of the cerebellum.
- The culture inserts were placed in the pre-arranged tissue culture plate and incubated immediately.

8.2. Experiments and maintenance of the cultures

- The incubation medium was changed every 2-3 days. Fresh incubation medium was pre-warmed to 37°C and the pH was adjusted to 7.2-7.4, if necessary.
- Drugs were added to the culture medium after every medium change.

8.2.1. Pharmacological inhibitors

Solvents for stock solutions (SS, sterile):

- Preparation medium (PM)
- Aqua bidest (H_2O)
- 1:1 mixture of ethanol and dimetyl sulfoxide (EtD)

All Stock solutions 1 (see Table 1) were kept at -20°C. Stock solutions 2 were kept at -20°C if the solvent was EtD, and at -4°C for the duration of the experiment if the solvent was PM or H_2O. Concentrations of stock solutions 2 were chosen according to the desired end-concentration for the experiment, as to make sure that the concentration of solvent in the culture medium would not exceed 1% (Kapfhammer, 2010).

Table 2: Pharmacological inhibitors and stock solutions

Pharmacological Inhibitors	MW [g/mol]	Stock solution 1	Stock solution 2	Dealer
ω-Agatoxin IVA	5202.48	0.1mM (1mg in 1.92 ml PM)	50 µM (dilute SS1 1:1 in EtD)	Smartox 11AGA001
Bepridil hydrochloride	421.02	100mM (50mg in 1.18ml in H_2O)	10mM (dilute SS1 1:9 in EtD)	Tocris 4117
Carboxyeosin (light sensitive)	873.05	50mM (5.0mg in 0.115ml in EtD)	10mM (dilute SS1 1:4 in EtD)	Invitrogen 196419
CB-DMB	472.37	50 mM (5 mg/ml in H_2O)	10 mM (dilute SS1 1:1 in EtD)	Sigma c-5374
ω-Conotoxin MVIIC	2750.20	1mM (0.1 mg in 36.36 µl EtD)	500 µM (dilute SS1 1:1 in EtD)	Smartox 08CON002
DHPG (RS)-3,5- Dihydroxyphenylglycine	187.67	30mM (10mg in 1.776ml PM)	5mM (dilute SS1 1:5 in EtD)	Tocris 0342
DL-AP5	197.13	100mM (10mg in 507µl PM)	50mM (dilute SS1 1:1 in EtD)	Tocris 0105
CNQX	232.16	50mM (10mg in 431µl PM)	20mM (dilute SS1 1:1.5 in EtD)	Tocris 0190
Gabazine	368.23	10mM (3.68 mg in 1 ml H_2O)	10mM (dilute SS1 0:0 in H_2O)	Abcam 144487
KB-R7943	418.4	100mM (10mg in 0.234 ml EtD)	10mM (dilute SS1 1:9 in EtD)	Tocris 600
Mibefradil	568.55	30mM (10mg in 568 µl in H_2O)	1mM (dilute SS1 1:29 in EtD)	Tocris 2168
PMA Phorbol 12-myristate 13-acetate	616.8	1.5 mM (0.925 mg/ml in EtD)	50µM (dilute SS1 1:59 in EtD)	Tocris 1201
ORM-10103	456.6	50mM (5 mg in 287.06 µl EtD)	10mM (dilute SS1 1:19 in EtD)	Sigma 3751
SN-6	402.9	25 mM (10 mg/ml in EtD)	5mM (dilute SS1 1:4 in EtD)	Tocris 1147
SEA0400	371.38	5mM (10.3mg in 554µl EtD)	100µM (dilute SS1 1:49 in EtD)	Taisho Pharma BP0376
YM-244769	469.15	4mM (1.88mg/ml in EtD)	1mM (dilute SS1 1:3 in EtD)	Tocris 1268

8.2.2. Pharmacological treatments to study dendritic development after PMCA2 and NCX inhibition

- All pharmacological treatments were started at DIV2-3 and maintained for 7 days, until the end of the culture period.
- To ensure a complete blockade of PMCA2, Carboxyeosin was added 24 hours before mGluR1 stimulation by DHPG and PKC activation by PMA.

- NCX inhibitors (Bepridil, KB-R7943, CB-DMB, ORM-10103, SEA0400, YM-244769 and SN-6 were individually added on 2nd or 3rd DIV.
- The P/Q-and T-type Ca^{2+} channel inhibitors (ω-agatoxin IVA, ω-conotoxin MVIIC and Mibefradil) and AMPA, NMDA and GABA-A receptor antagonist (CNQX, DL-AP5 and Gabazine) were added simultaneously with NCX inhibitors as per the experimental conditions (McDonough *et al.*, 2002, Gugger et al., 2012).

8.3. Immunohistochemistry

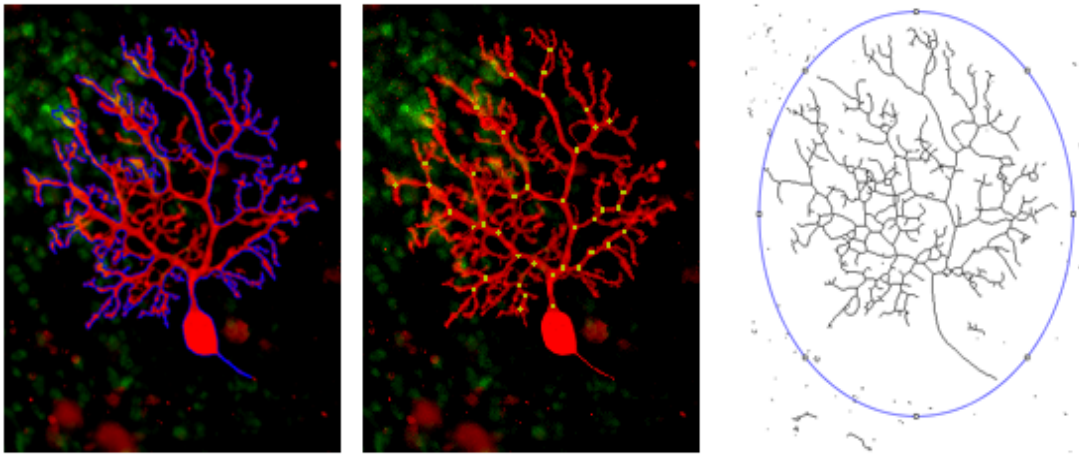
- At the end of the culture period, cultures were fixed in 4% paraformaldehyde in 100 mM phosphate buffer (PB) for 6 hours at 4 °C.
- The slices were washed with PB 3 times for 10 min.
- The plastic feet at the culture inserts were cut off to limit the required amount of antibody solution to 800 µl.
- The primary antibody solution was prepared in PB as follows:
    - 0.5 % Triton X-100, in order to permeabilize the tissue and prevent non-specific antigen binding.
    - 3 % normal goat serum, in order to block non-specific antigen binding
    - rabbit anti-Calbindin D-28K (Swant) 1:1000, to visualize Purkinje cells in dendritic development studies.
    - monoclonal mouse anti-NeuN (Chemicon, Millipore) 1:500, to visualize CGNs
    - rabbit anti-PMCA2 (Abcam) 1:1000, to visualize plasma membrane calcium ATPase 2 in Purkinje cells
    - rabbit anti-Slc8a1 (Alomone) 1:100, to visualize sodium-calcium exchanger (NCX1)
    - rabbit anti-Slc8a2 (Origene) 1:300, to visualize sodium-calcium exchanger (NCX2)
    - rabbit anti-Slc8a3 (Origene) 1:1000, to visualize sodium-calcium exchanger (NCX3).
    - mouse anti-Calbindin D-28K (Swant) 1:500, to visualize Purkinje cells in NCX isoform expression studies.
    - guinea pig anti-Calbindin D-28K (SYSY) 1:500, to visualize Purkinje cells in NCX isoform triple staining expression studies.
    - mouse anti-Parvalbumin (Swant) 1:5000, to visualize molecular interneurons for NCX isoform expression studies.
- The slices were incubated with primary antibody solution at 4 °C overnight.
- The slices were washed with PB 3 times for 10-20 min.

- The secondary Antibody solution was prepared in PB as follows:
    - 0.1 % Triton X-100, in order to prevent non-specific antigen binding
    - goat anti-rabbit Alexa 568 (Molecular Probes, Invitrogen) 1:500
    - goat anti-mouse Alexa 488 (Molecular Probes, Invitrogen) 1:500
    - goat anti-mouse Alexa 405 (Abcam, United Kingdom) 1:500
- The slices were incubated in secondary antibody solution at room temperature for 1-4 hours.
- The slices were washed with PB 3 times for 10 min.
- Stained slices were mounted on glass slides (Thermo Scientific Menzel-Gläser Superfrost Plus, Art. No. J1800AMNZ) with coverslip (VWR 24x50 mm) in Vectashield, Hardset mounting medium (H-1400).
- Cultures were observed under an Olympus AX-70 microscope equipped with a Spot digital camera and images were made at 20x lens for analysis.
- For NCX expression studies, confocal microscopy was performed on an upright laser scanning microscope (Zeiss LSM700) equipped with solid-state lasers. Images were acquired using a Plan Apo N 63× 1.4 NA oil immersion objective (Zeiss) and standard PMT detectors. Optical z-sections were separated by 200 nm. The laser lines 488 and 568 nm were used for excitation. Multichannel imaging was achieved through sequential acquisition of wavelengths frame by frame.
- Recorded images were further processed for brightness and contrast with Adobe Photoshop image processing software.

8.4. Quantitative analysis of cultured Purkinje cells

- Slice cultures were observed under Olympus AX-70 microscope equipped with a Spot Insight digital camera.
- Isolated and non-overlapping Purkinje cells with a well-defined dendritic arbor were chosen for analysis.
- Purkinje cell dendritic branch points were counted manually by using Adobe Photoshop software.
- The image analysis program Image Pro Express was used to trace the outline of the Purkinje cell dendritic trees by selecting the area using the magic wand tool. This yielded the area covered by the dendritic tree.

- The dendritic length was measured by Image J software by converting image to binary and then skeletonizing it. The defined skeleton was measured as number of pixels. These pixels were converted in to micrometer (2.71pixels=1 micrometer).
- Cells were acquired from three independent experiments with an average number of 20 or more cells per experiment.



dendritic area: 27023 dendritic branchpoints: 38 dendritic length: 4764

Figure 17: Dendritic measurements for Purkinje cells: The dendritic area of isolated and non-overlapping Purkinje cell was measured by Image Pro Express software as mentioned above. The dendritic area measured in pixels (e.g. 27023 pixels for the given cell (A). The dendritic length was measured by using ImageJ free software tool by inverting and skeletonizing image. The number of values were in pixels (e.g. 4764). The dendritic branchpoints were counted manually on Adobe Photoshop (e.g. 38).

8.5. Statistical Analyses

- The data were analyzed using GraphPad Prism 7.0 software.
- The statistical significance of differences in parameters was assessed by non-parametric analysis of variance (Kruskal-Wallis test) followed by Dunn's post test. For comparisons of single data columns, Mann-Whitney's non-parametric test was used. Confidence intervals were 95 %, statistical significance with $p<0.05$.

The mean value of the dendritic tree area, dendritic length and number of branch points of untreated control cells were set to 100 % and the results were expressed as percentage of controls.

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